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Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells

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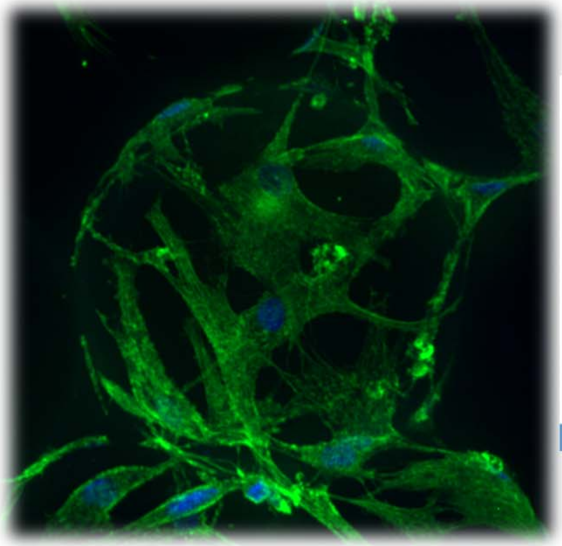

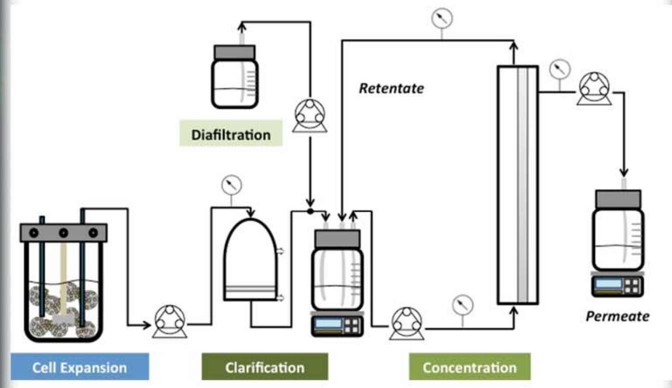

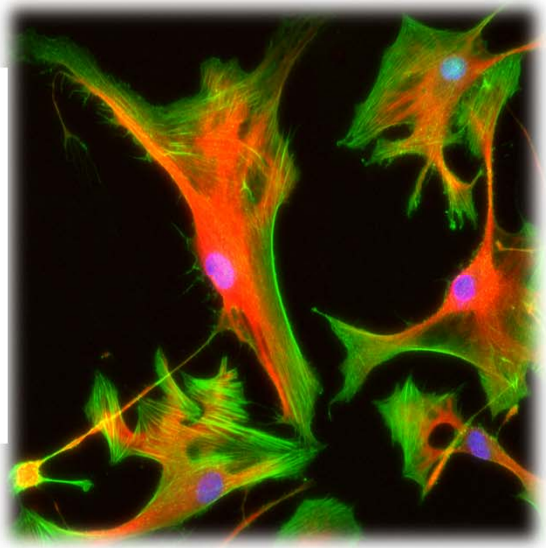

Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells

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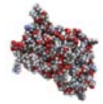
Integrated Continuous Manufacturing II | November, 4, 2015

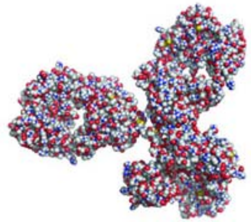

iBET
Oeiras, Portugal

- Cell-Based Therapies: Increased complexity

Small Molecule
(e.g. aspirin)



Recombinant protein
(e.g. human Growth Hormone)



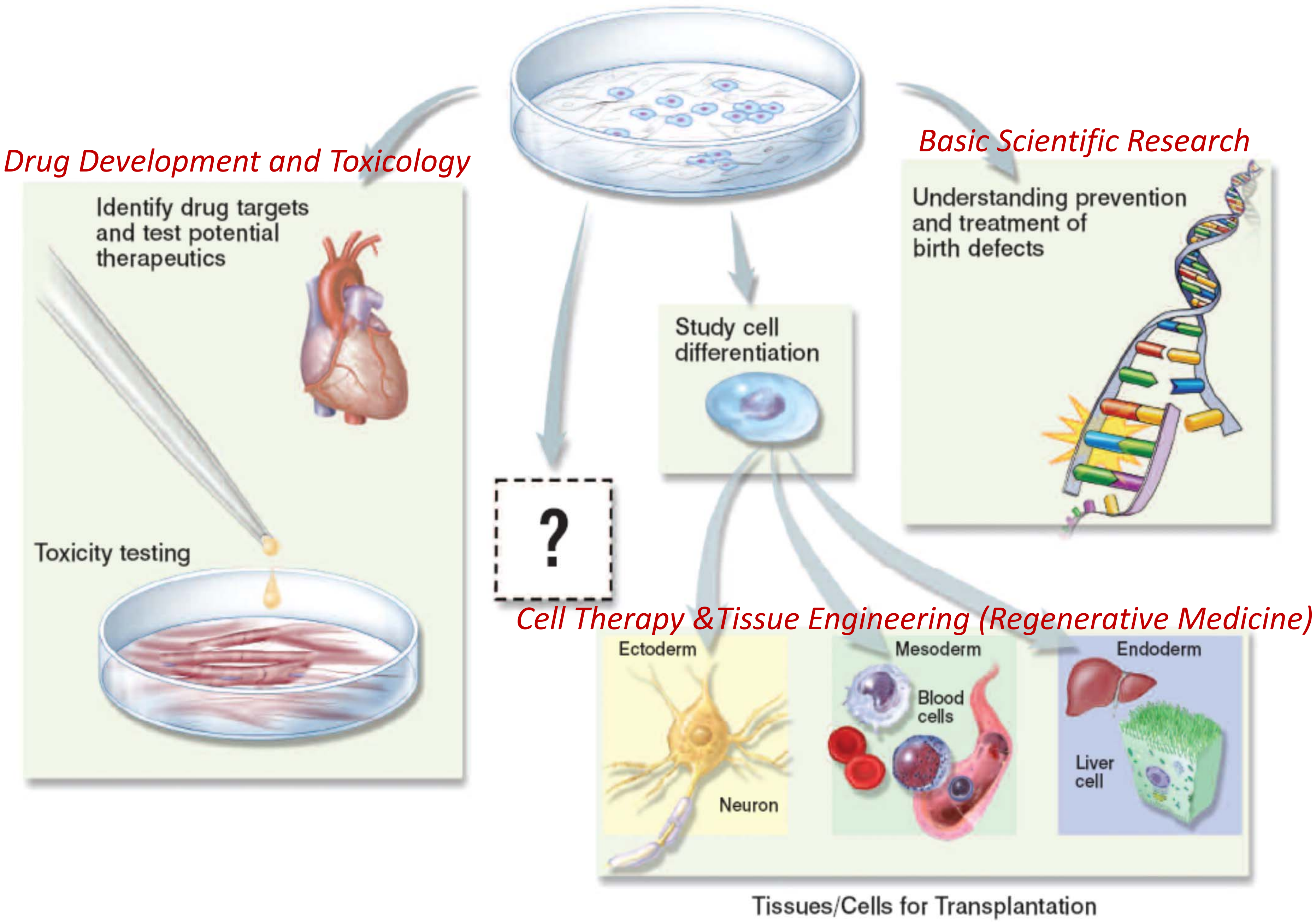
Monoclonal Antibody
(e.g. IgG1)

Differences in mass by ~ $1\text{x}10^{12}$

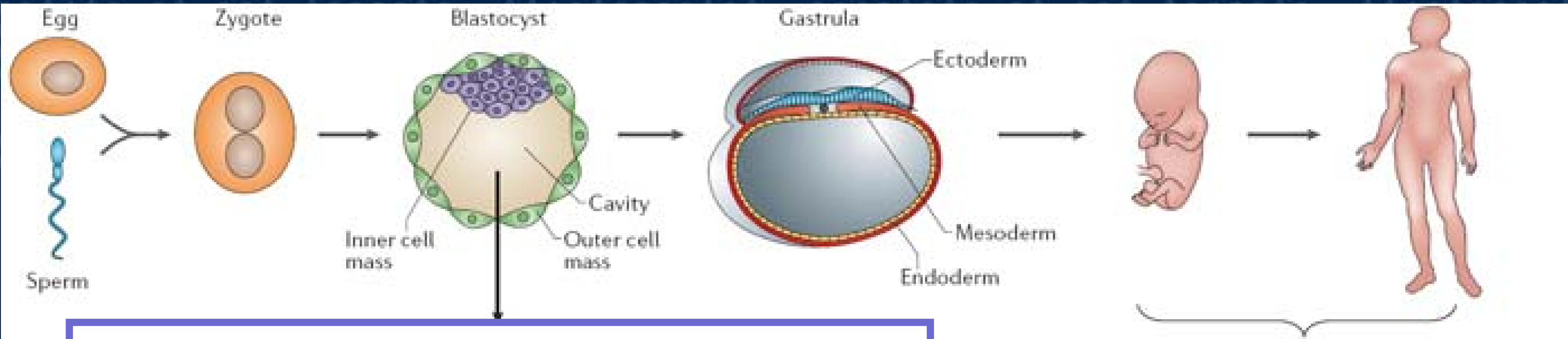
in surface area by ~ $5\text{x}10^{6}$

Courtesy: G. Russotti, Celgene Cellular Therapeutics

The Promise of Stem Cell Research

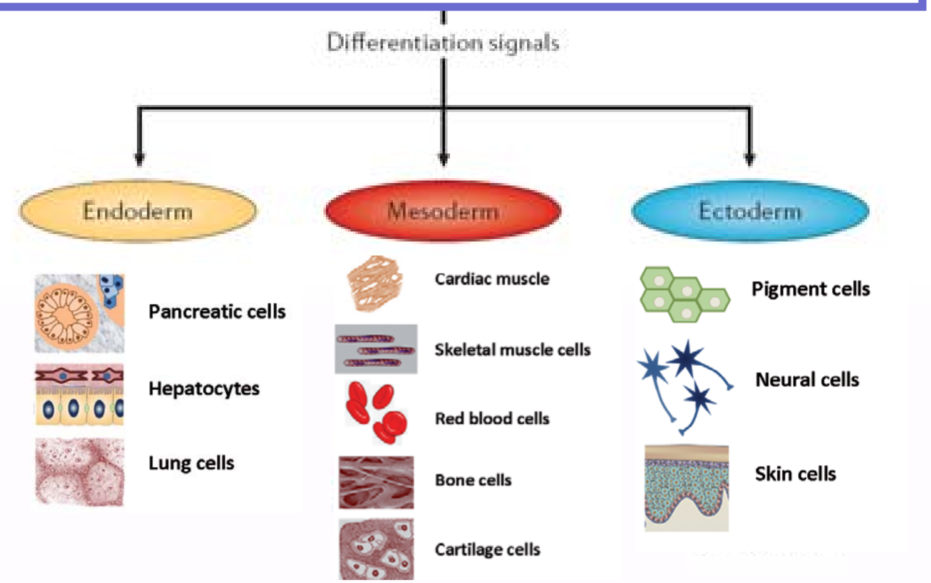


Types



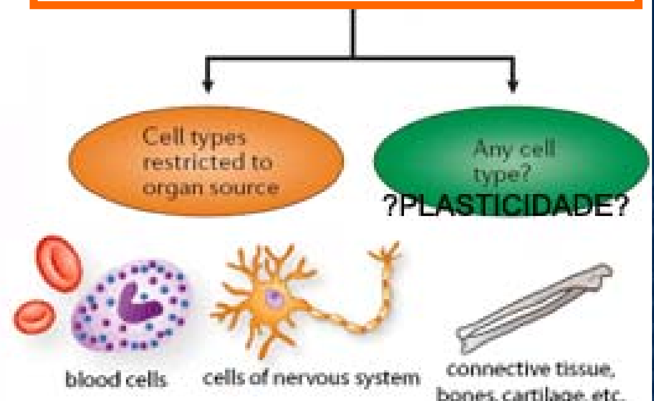
Embryonic Stem Cells (ESCs)

undifferentiated cells derived from a preimplantation embryo (inner cell mass of blastocyst) that are capable of self-renewal, and can develop into cells and tissues of the three primary germ layers



Adult Stem Cells (ASCs)

undifferentiated cells, found in tissues or organs of the body after embryonic development, that are capable of self-renewal and differentiate into specialized cells to replenish dying cells and/or regenerate damaged tissues



Adaptado de O'Connor & Crystal 2006, Nature Reviews Genetics 7, 261-276

Induced Pluripotent Stem Cells (iPSC)

Yamanaka's Lab (Japan)

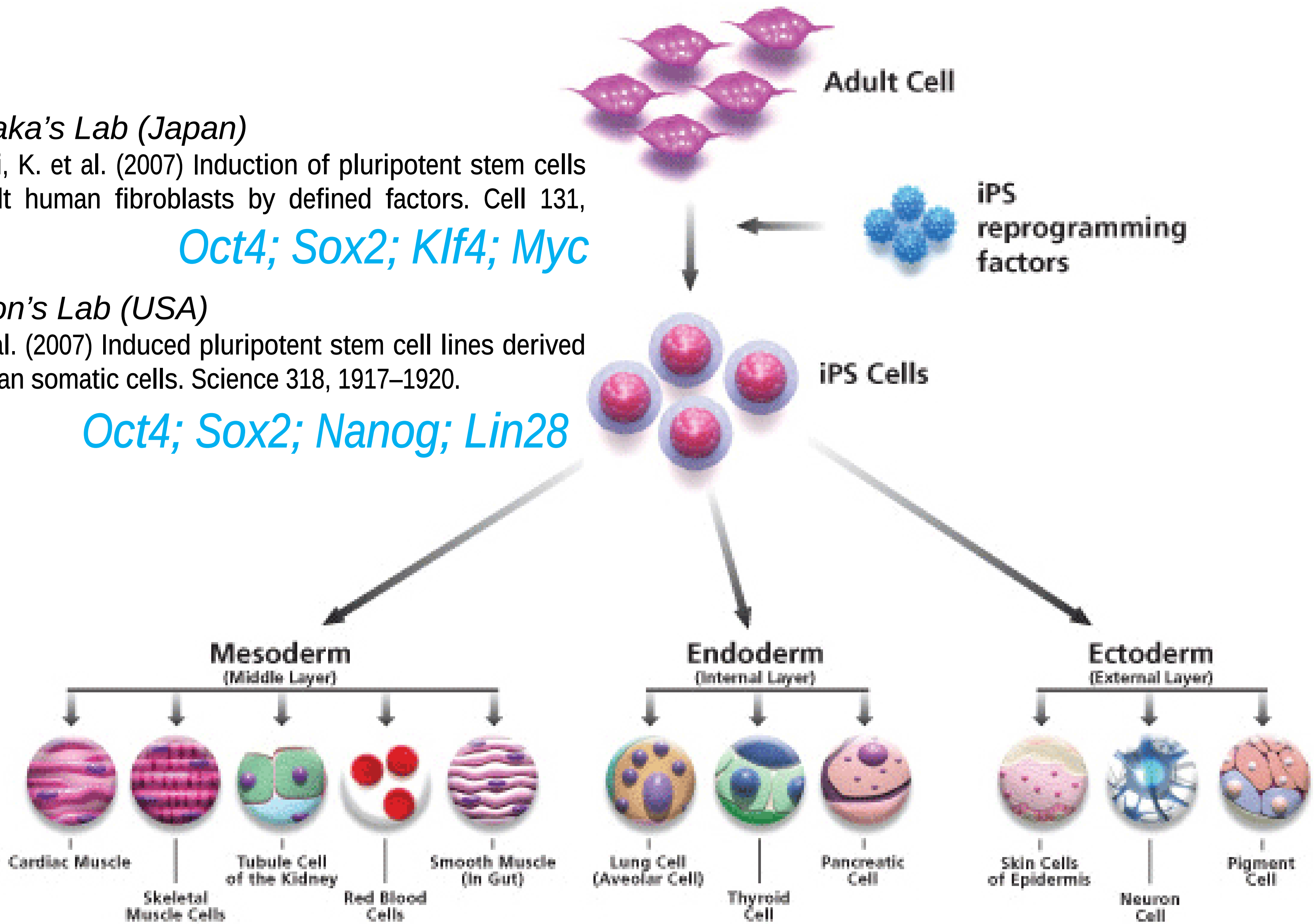
Takahashi, K. et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872

Oct4; Sox2; Klf4; Myc

Thomson's Lab (USA)

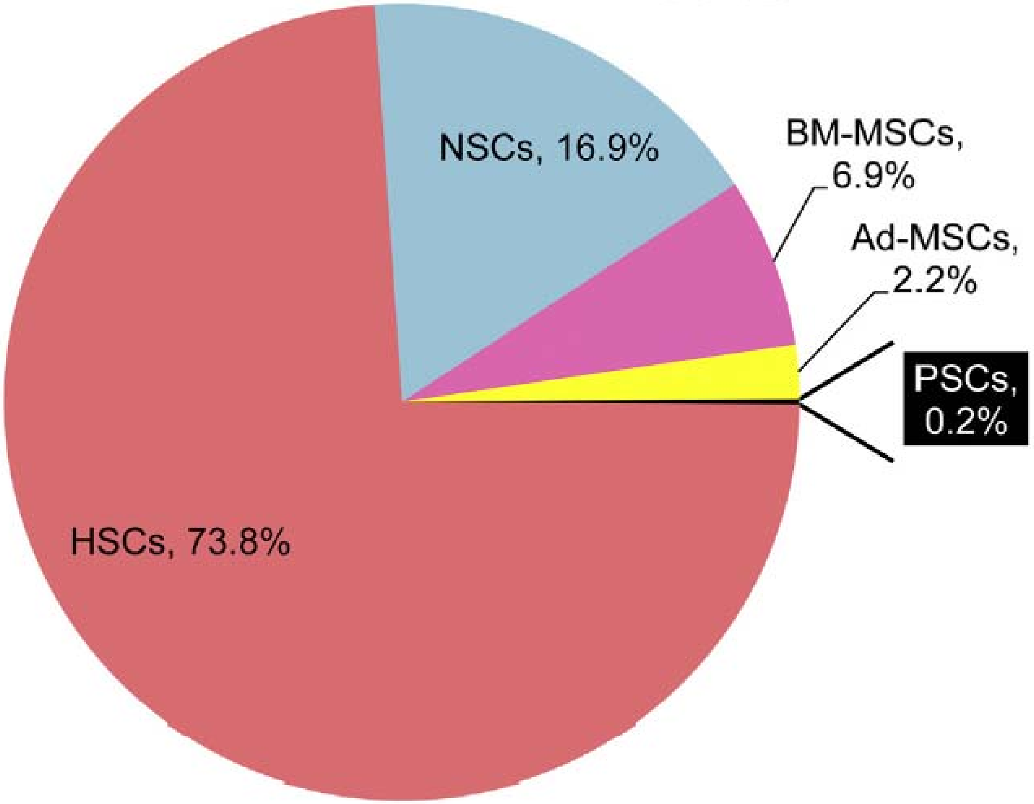
Yu, J. et al. (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917–1920.

Oct4; Sox2; Nanog; Lin28



Clinical trials

Global stem cell based clinical trials: 1750



Mesenchymal

Hematopoietic

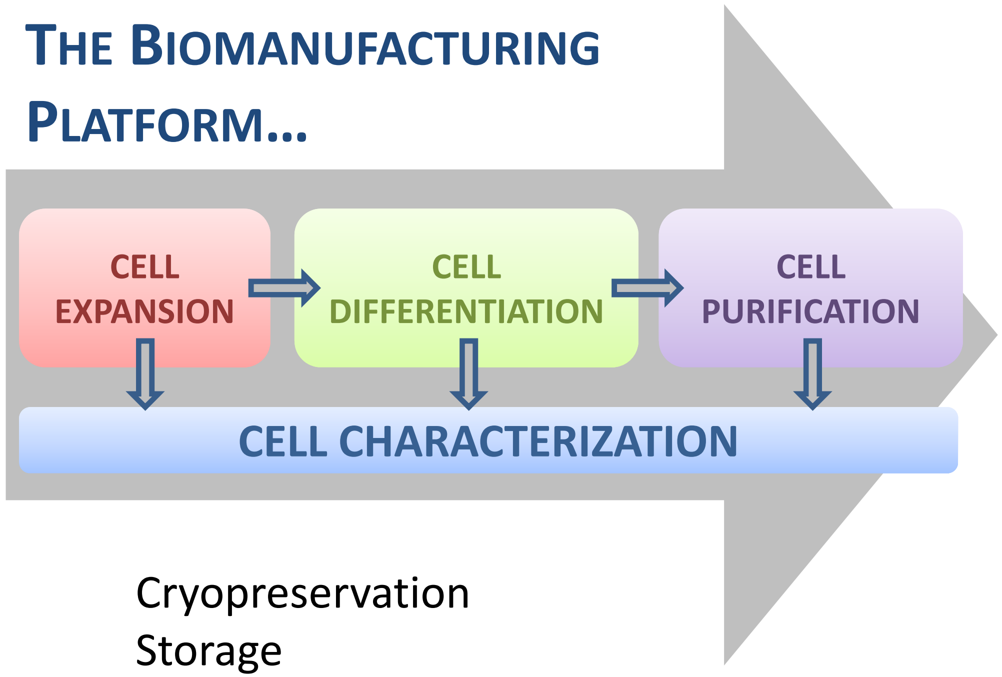
Fig. 1. The share of hPSC based clinical trials in global stem cell based clinical trials at 2012. Pie chart indicating the relative percentage of pluripotent stem cells based clinical trials in total number of open global stem cell based clinical trials (1750 trials) compared to other major stem cell types including hematopoietic (HSCs), neural (NSCs), bone marrow-derived mesenchymal stem cells (BM-MSCs), and adipose-derived MSCs (Ad-MSCs), as extracted from the U.S. NIH website: www.clinicaltrials.gov.

Abbasalizadeh S. and Baharvand *H, Biotechnology Advances 2013*

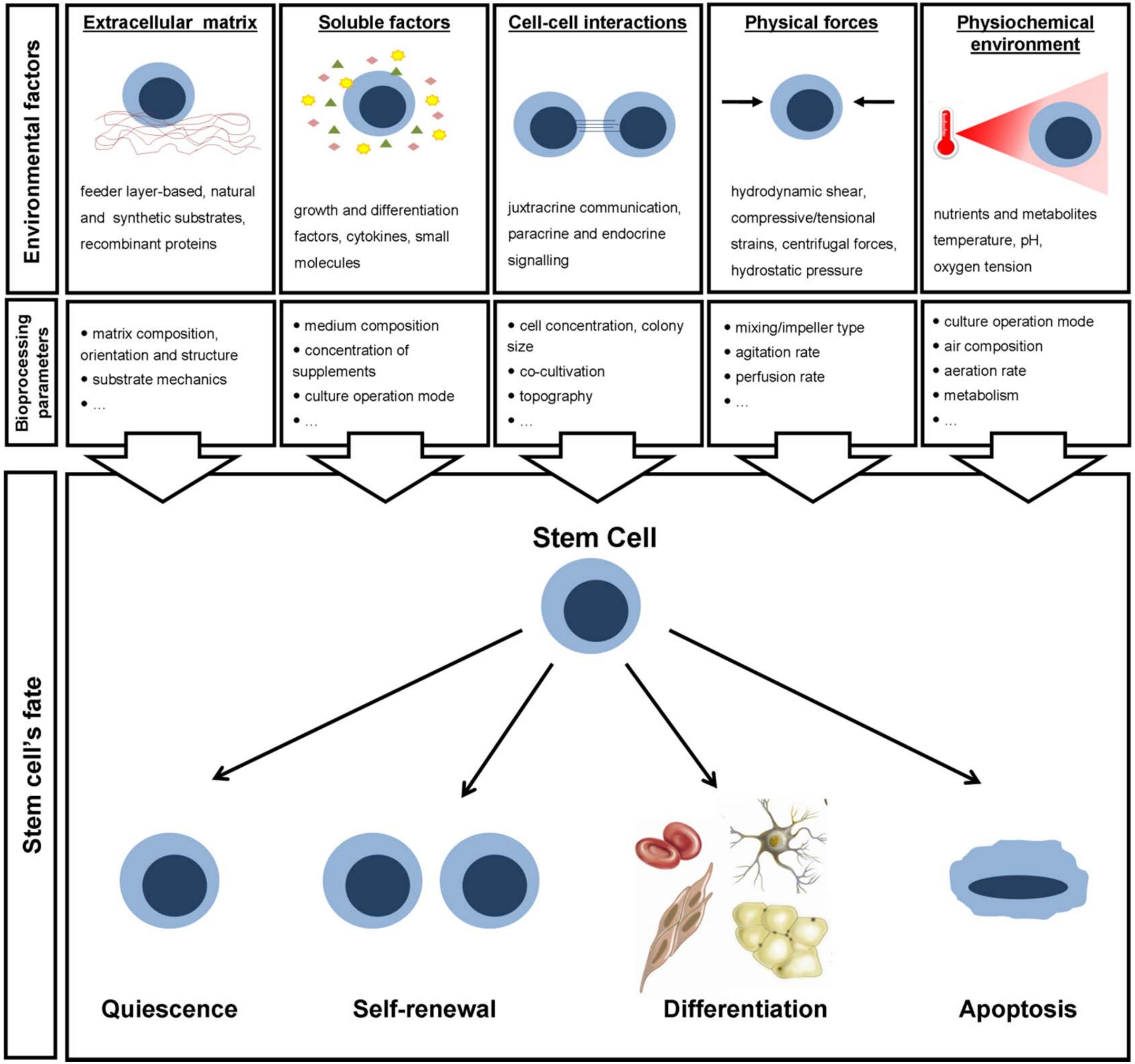
Develop a robust and integrated platform for the production and characterization of challenging stem cell-based products

The Product …

The Biomanufacturing Platform…



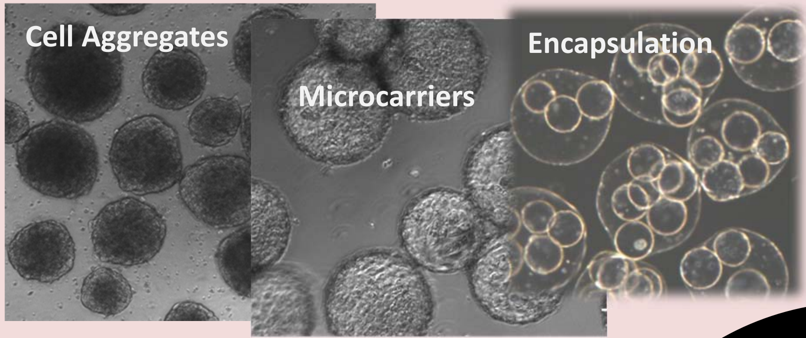
Cryopreservation
Storage
Transportation



Serra M. et al (2012) Trends in Biotechnology

Strategy

3D Culture Systems



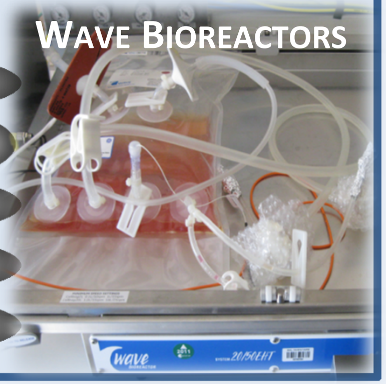
- ✓ Improve cell viability and function
- ✓ Enhance proliferation/differentiation capacity
- ✓ Preserve 3D cell-cell and cell-matrix contacts
- ✓ Reproducibility and predictability

Bioreactors

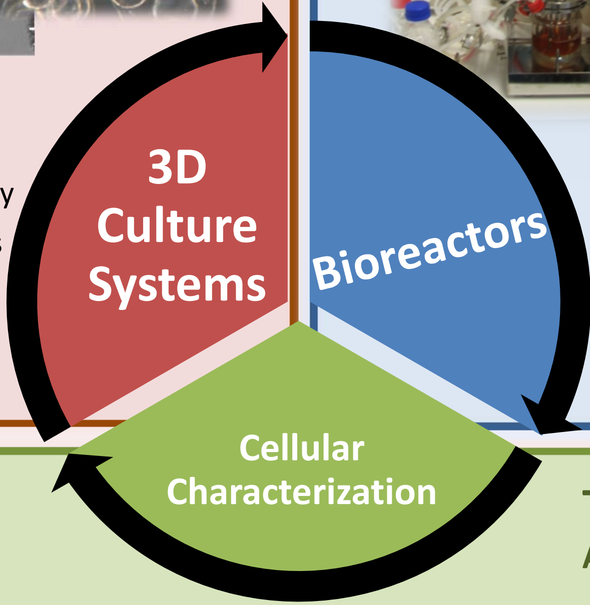
Stirred Tank Bioreactors

- ✓ Scalability
- ✓ Straightforward operation
- ✓ Culture homogeneity
- ✓ Automated
- ✓ Reproducibility
- ✓ Non-destructive sampling
- ✓ cGMP

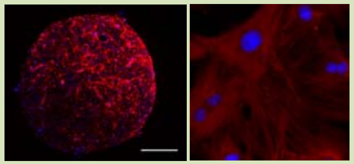
Wave Bioreactors



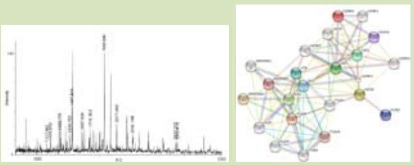
Temperature
pO_2 pH
Agitation rate
Perfusion
Gas composition



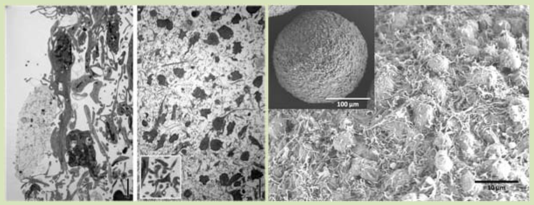
Phenotypic Analysis



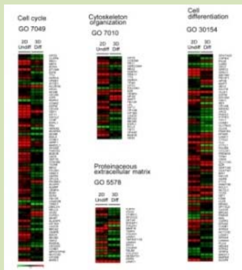
Proteomic Analysis



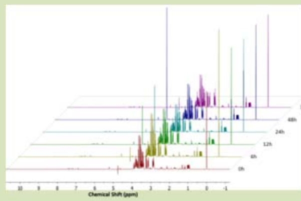
Ultrastructure Analysis



Transcriptomic Analysis

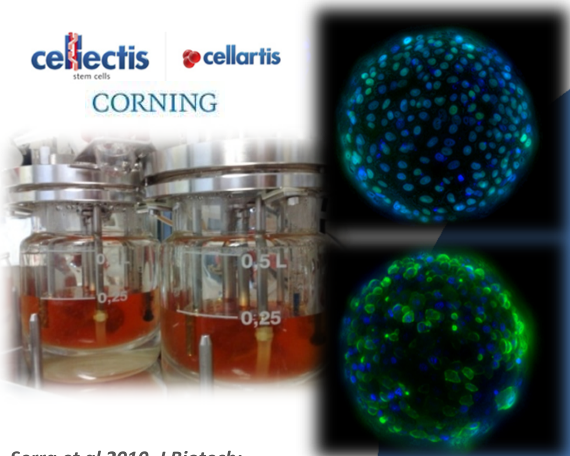


Metabolomic Analysis



Bioprocessing of Human Stem Cells for clinical and Pre-clinical res

Gomes-Alves et al 2014, Proteomics



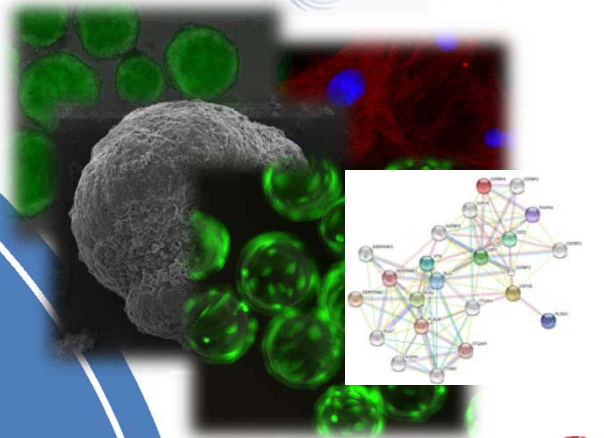
Serra et al 2010, J Biotech;
Serra/Correia et al 2011, PLoS ONE;
Silva et al 2015, Stem Cells Transl Med)

Clinical-grade expansion of hESCs

Production, Purification & Characterization of Cardiomyocytes derived from CSC & iPS cells

Neural differentiation of hNSCs

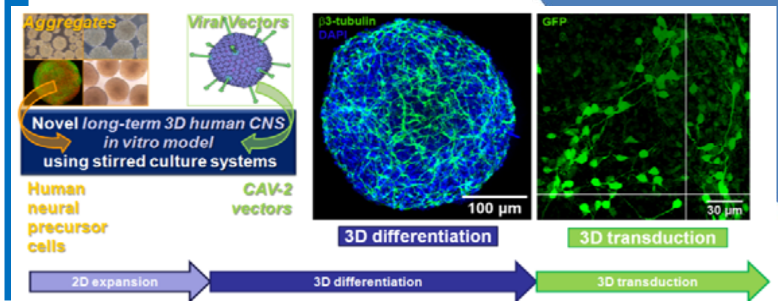
Up- and Down-stream processing of hMSCs

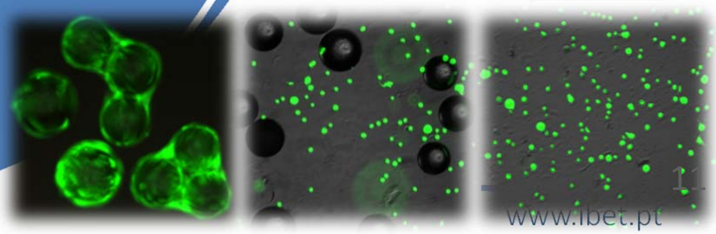


Serra et al 2012, Trends in Biotech;
Correia et al 2014, Stem Cell Rev Rep;

Cunha et al 2015, J Membr Sci;
Cunha et al 2015, J Biotech;
Sousa et al 2015, Biotechnol Prog

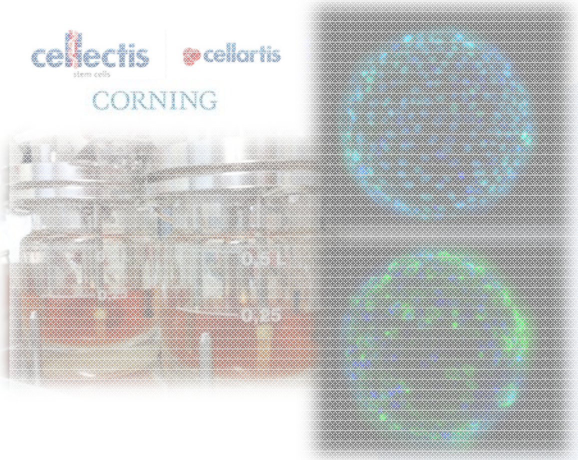
G. Schiavo ***Cancer Research UK;***
J. Schwarz ***U Leipzig Germany,***
E.J. Kremer ***GMM, France***





- Bioprocessing of Human Stem Cells for clinical application @ iBET

Gomes-Alves et al 2014, Proteomics



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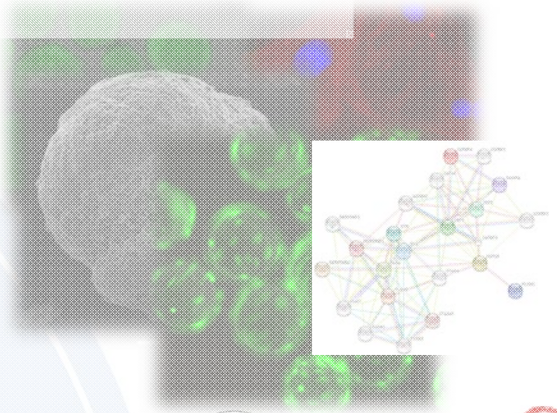

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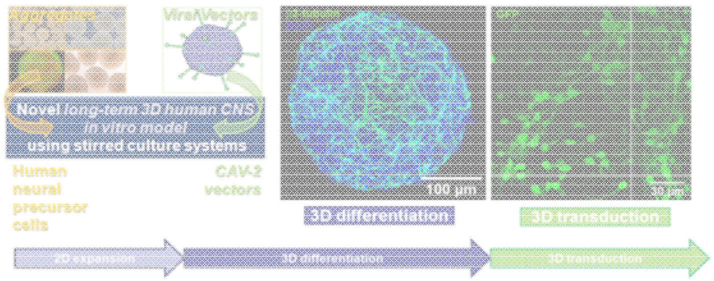
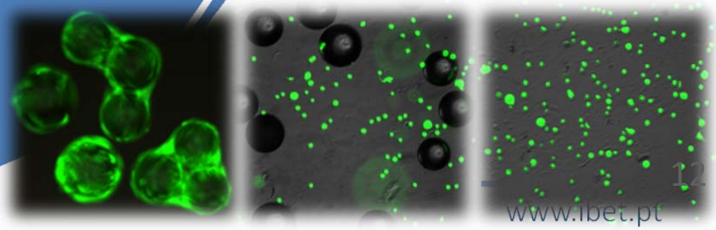
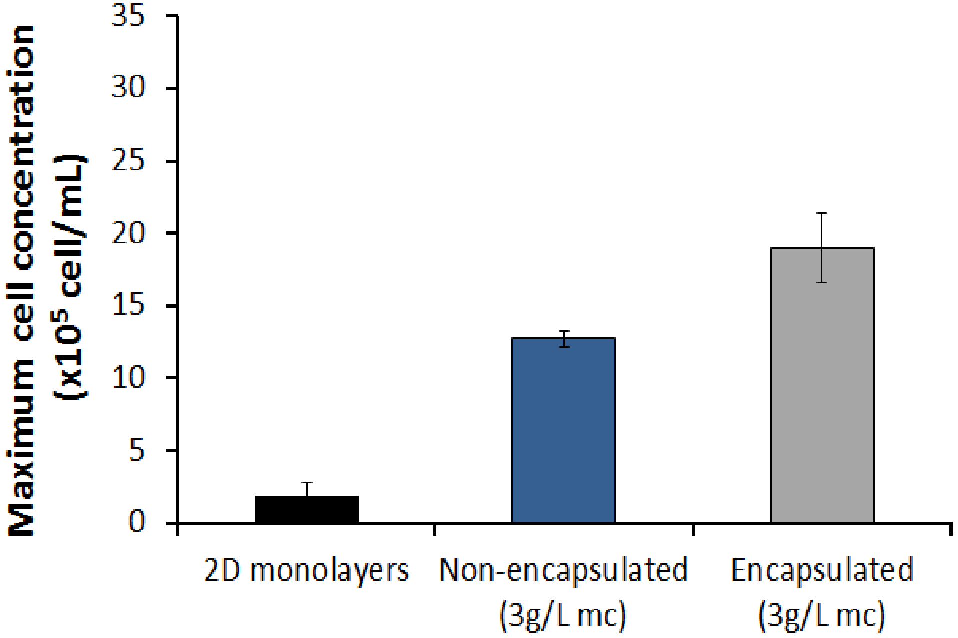




EXPANSION OF HESC IN ST BIOREACTORS: MAJOR ACHIEVEMENTS

Expansion



Higher Volumetric Productivities (up to 10x)

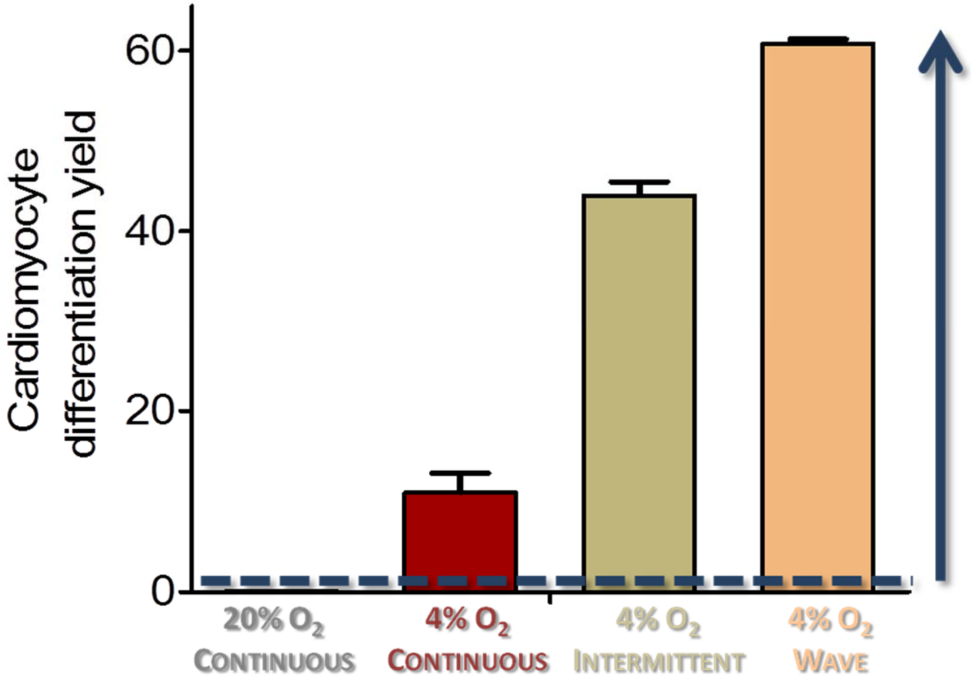
avoiding spontaneous differentiation

Serra/Correia et al., 2011, PloS ONE 8 (6)

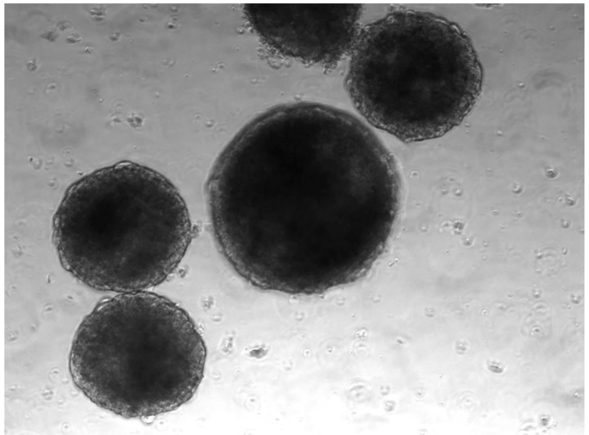
- Production of iPSC-Derived CMs: Major Achievements

The use of environmentally controlled bioreactors is critical to ensure ***EFFICIENT*** and ***SCALABLE*** production of functional cardiomyocytes derived from induced pluripotent stem cells

$$\text{Cardiomyocyte Differentiation Yield} = \frac{\text{No. of CMs}}{\text{No. of iPS cells}}$$



Correia *et al.*, 2014



>1000-FOLD IMPROVEMENT

2.3 x10^9 CMs

per 1L bioreactor run

human 3D neural models @ iBET : Strategy

Human Neural Cell Sources:

Human Embryonic Stem Cells (hESC) &
Embyonal Carcinoma Stem Cells

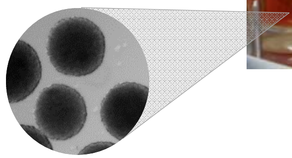
Human fetal Neural Stem Cells (hNSC)

Induced Pluripotent Stem Cells (iPSC)

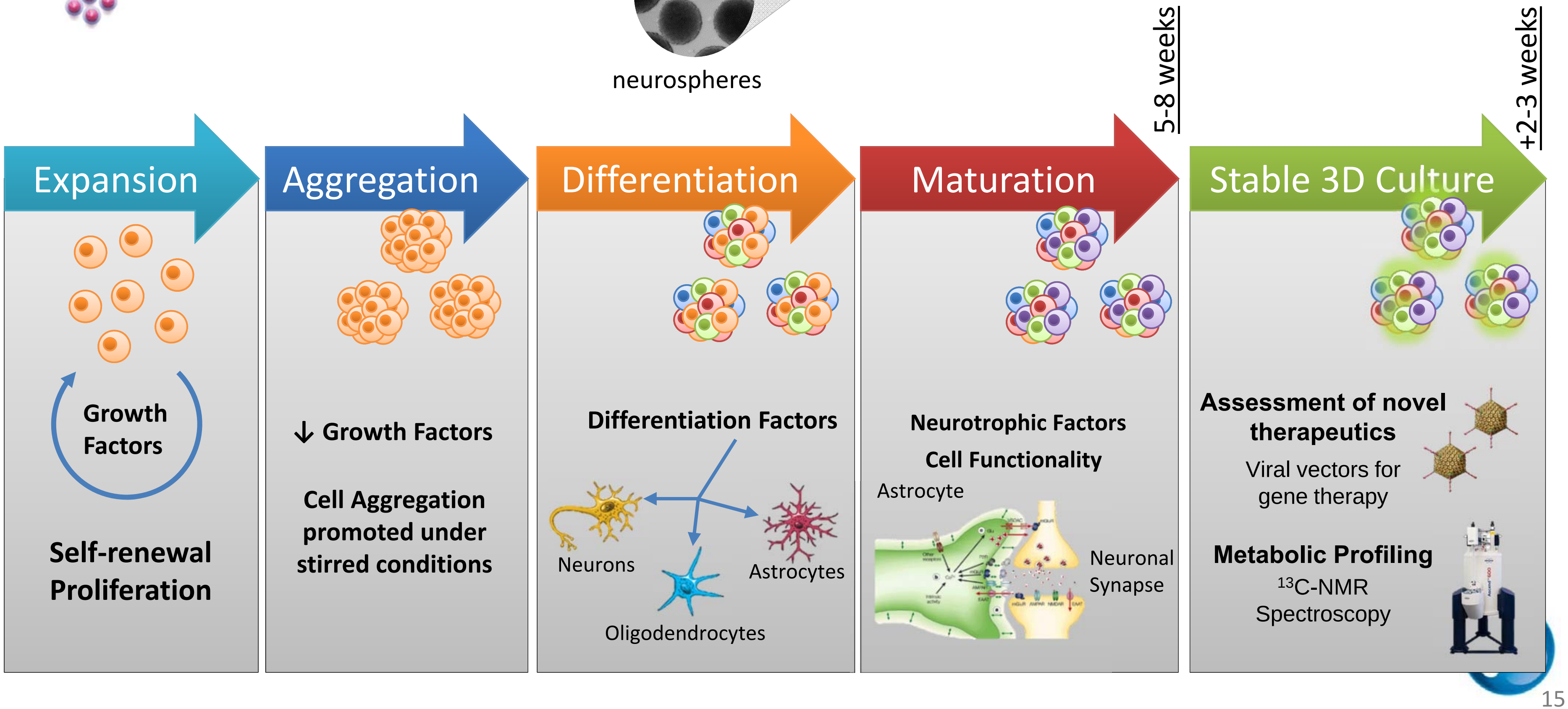
Continuos Perfusion Stirred Tank Bioreactors:

Systems: DasGip, BIOSTAT Qplus (Sartorius)

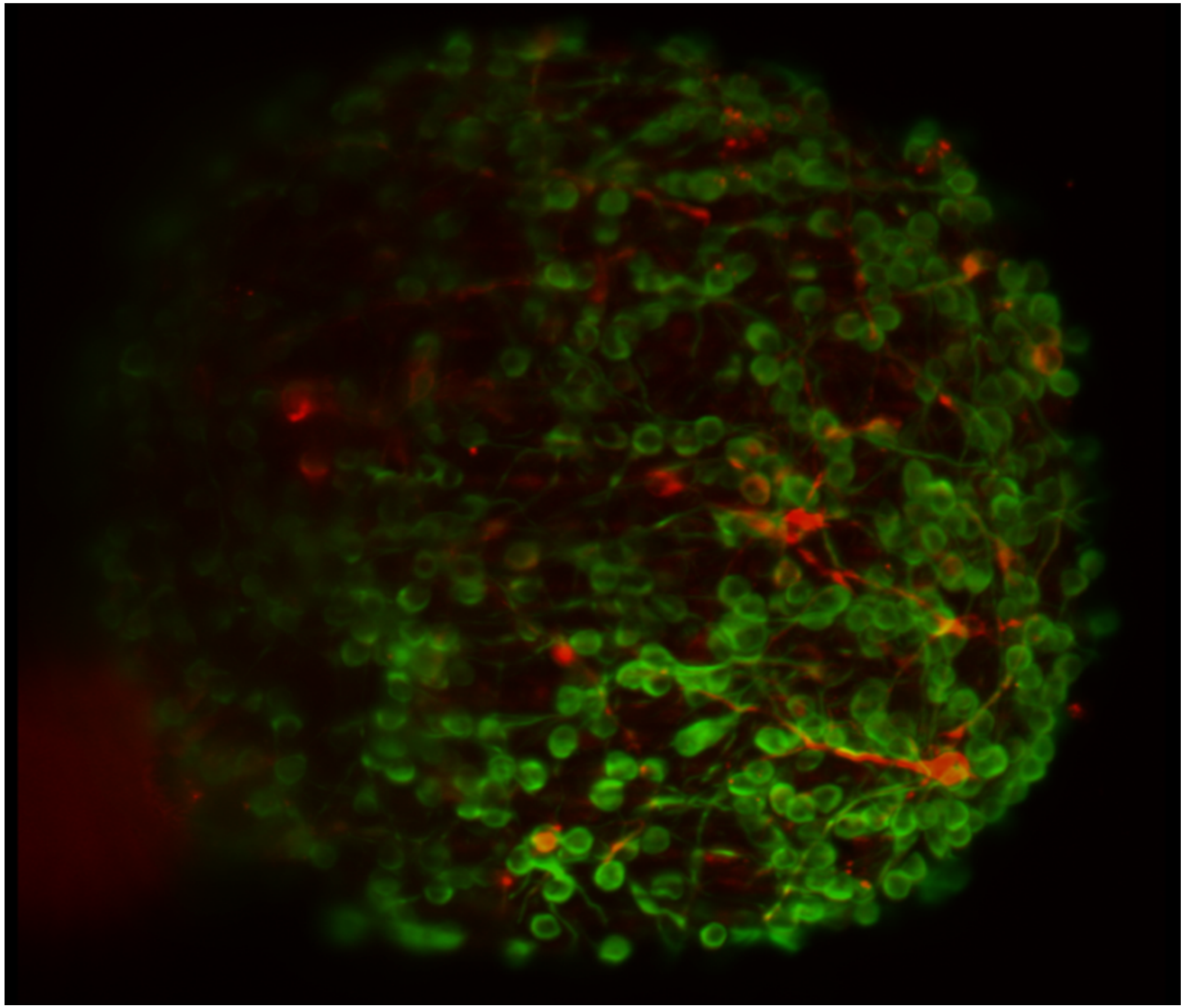
- **Working volumes:** 100-500 mL
- **Low Oxygen Tensions:** pO_2=15-25% (Air Saturation)



neurospheres



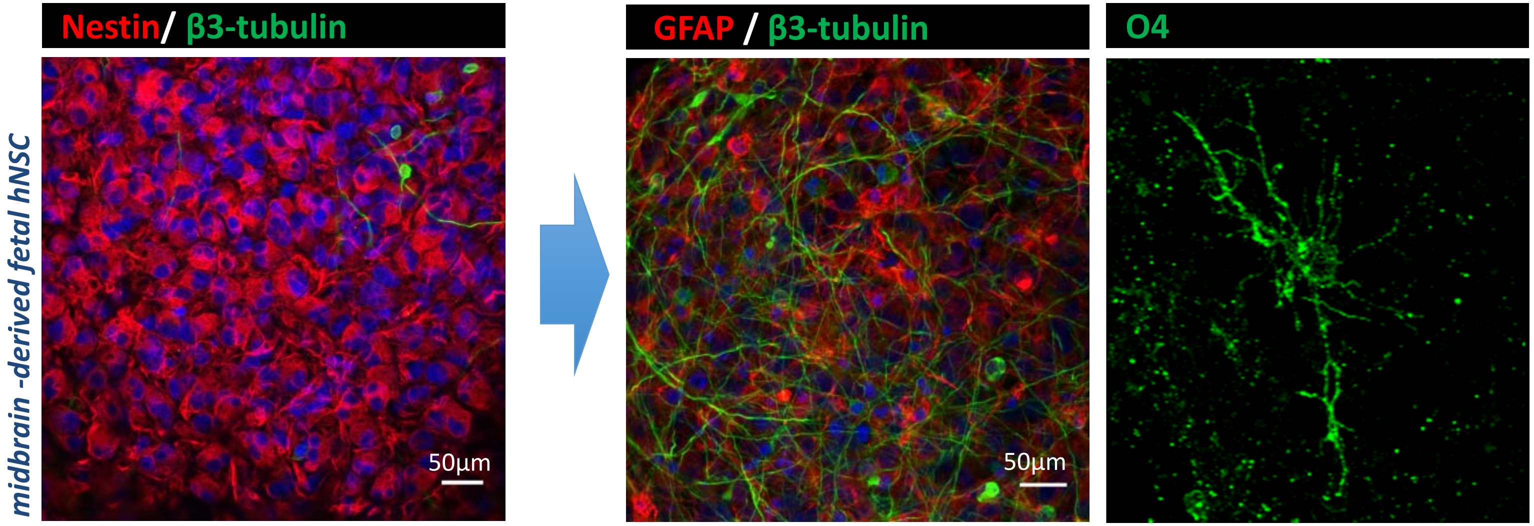
- Neuronal Dopaminergic Differentiation in 3D



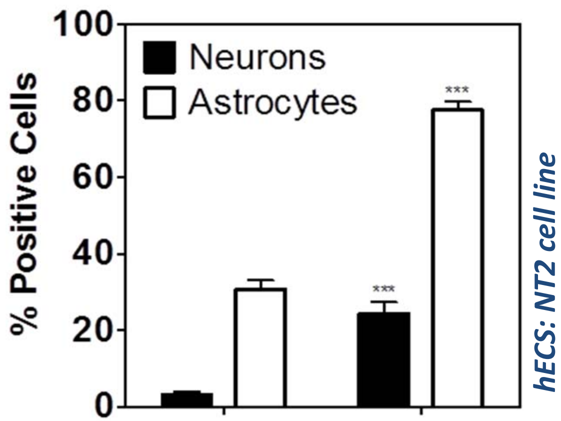
- ✓ Differentiated neurospheres enriched in <u>DOPAminergic neuronal markers</u>

βIII-tubulin
TH – Tyrosine Hydroxylase

Neuronal and glial 3D differentiation



Differentiation Yield



Differentiation	2D	3D
Time required (weeks)	10	5

Enrichement in cells from the **3 neural lineages**:

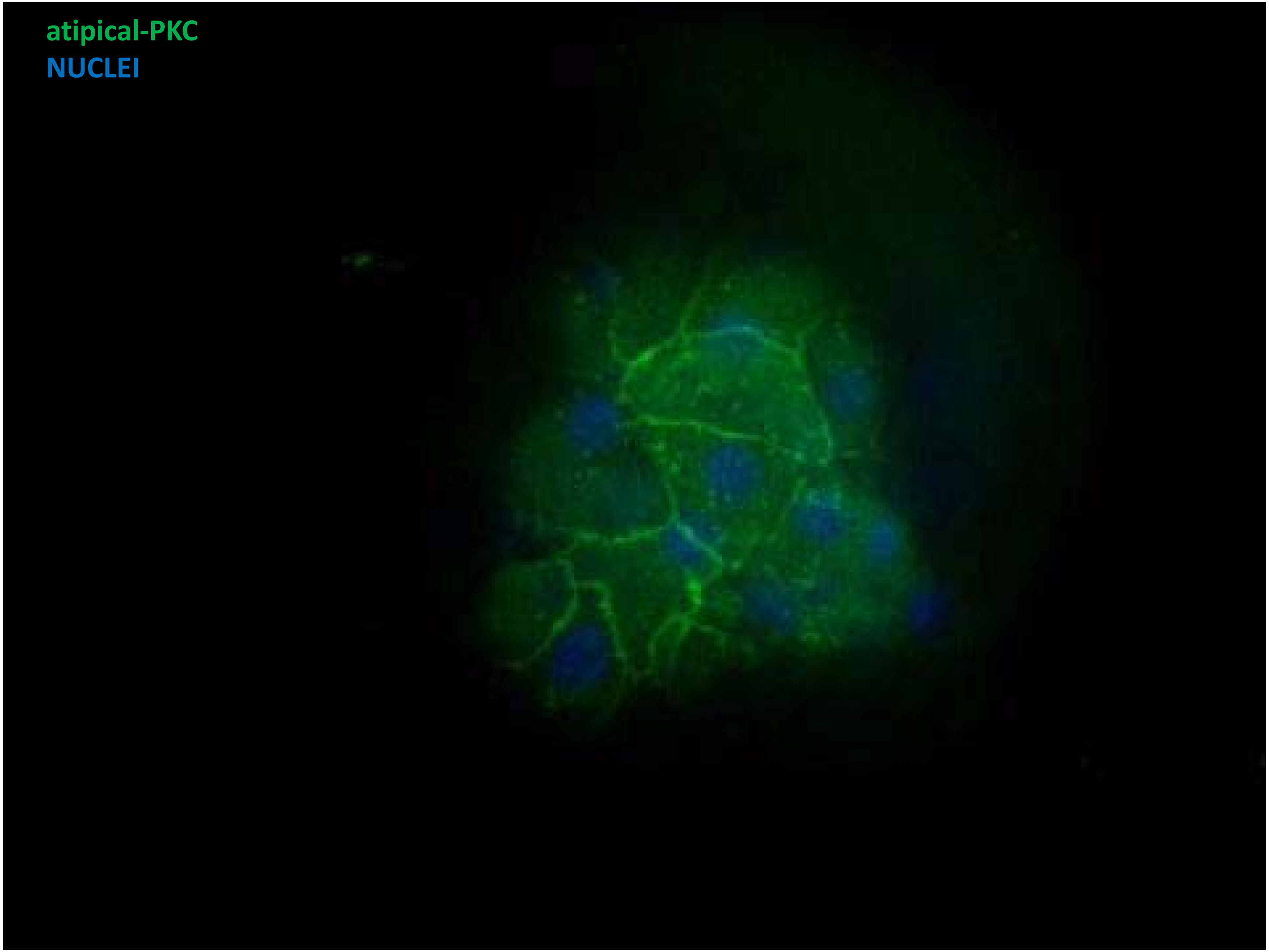
- ✓ **Neurons (βIII-tub)**
- ✓ **Astrocytes (GFAP)**
- ✓ **Oligodendrocytes (O4)**

3D differentiation process:

- ✓ **Higher differentiation efficiency**
- ✓ **Less time consuming process**

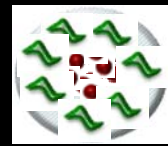
Simão *et al. (2014), Tissue Eng. Part A*; Terrasso *et al.* (2015) *J. Biotech.*

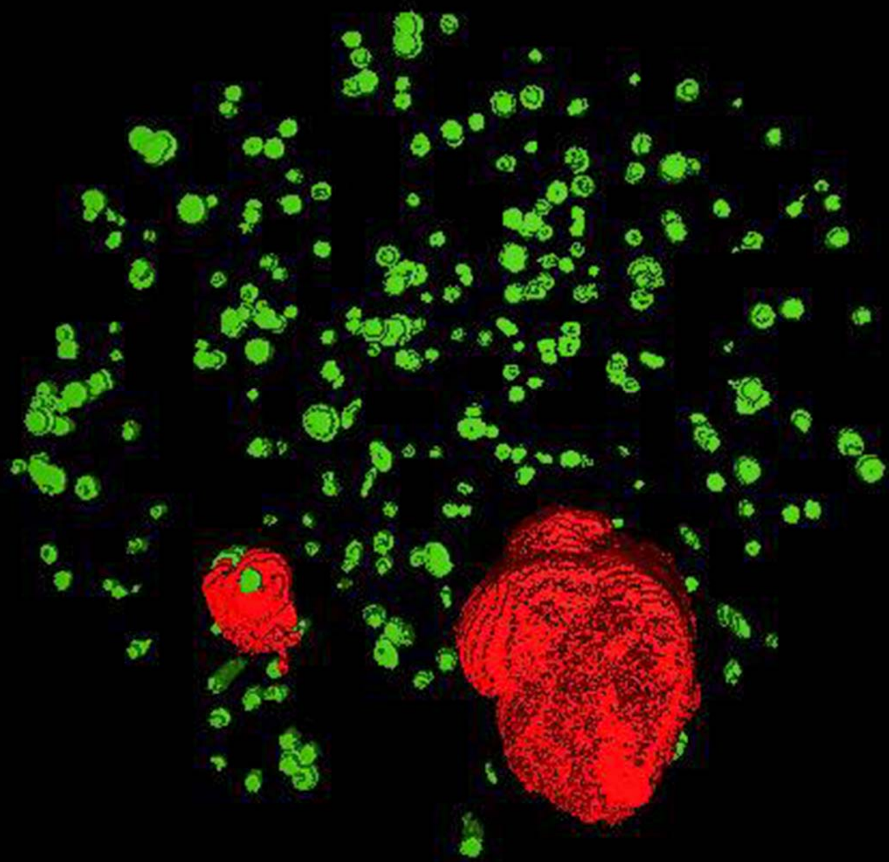
Human Hepatocytes perfused for 8 weeks in STB



MICROENVIRONMENT RECAPITULATION: ENCAPSULATION

Co-culture of tumour spheroids with fibroblast





- ✓ **in vivo-like tissue organization**: fibroblast are distributed around tumour aggregates

  Light sheet fluorescence microscopy (LSFM)

- Outline – Upstream and DSP og hMSC

i. Introduction (Biomanufacturing challenges)

ii. Goals

iii. Results - USP of hMSC :

  i. Overview

  ii. Continuous Perfusion and Semi-continuous operation modes

  iii. hMSC's Secretome

iv. Results - DSP of hMSC :

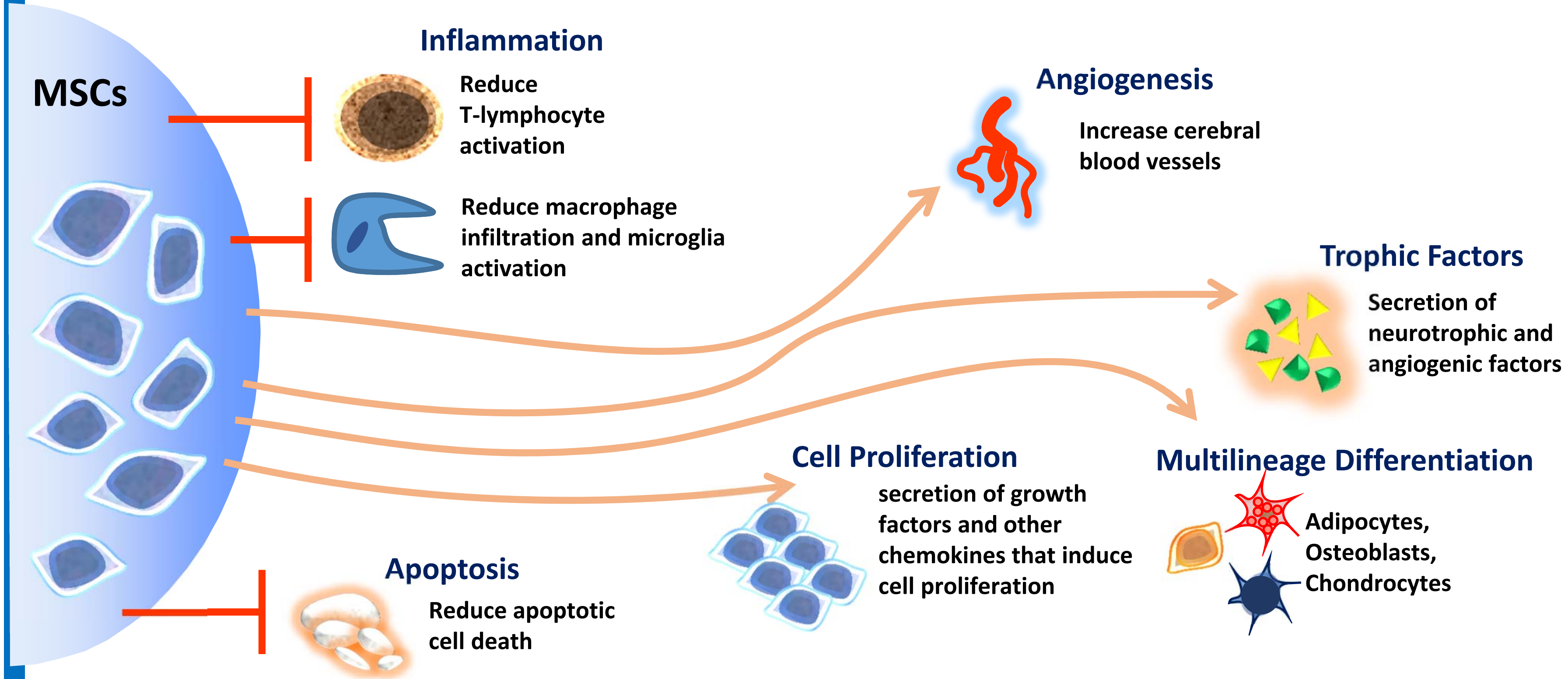
  i. Overview

  ii. Cell concentration

  iii. Cell washing

  iv. Process integration

Human Mesenchymal Stem Cells (hMSC) have become key candidates for autologous and allogeneic therapies

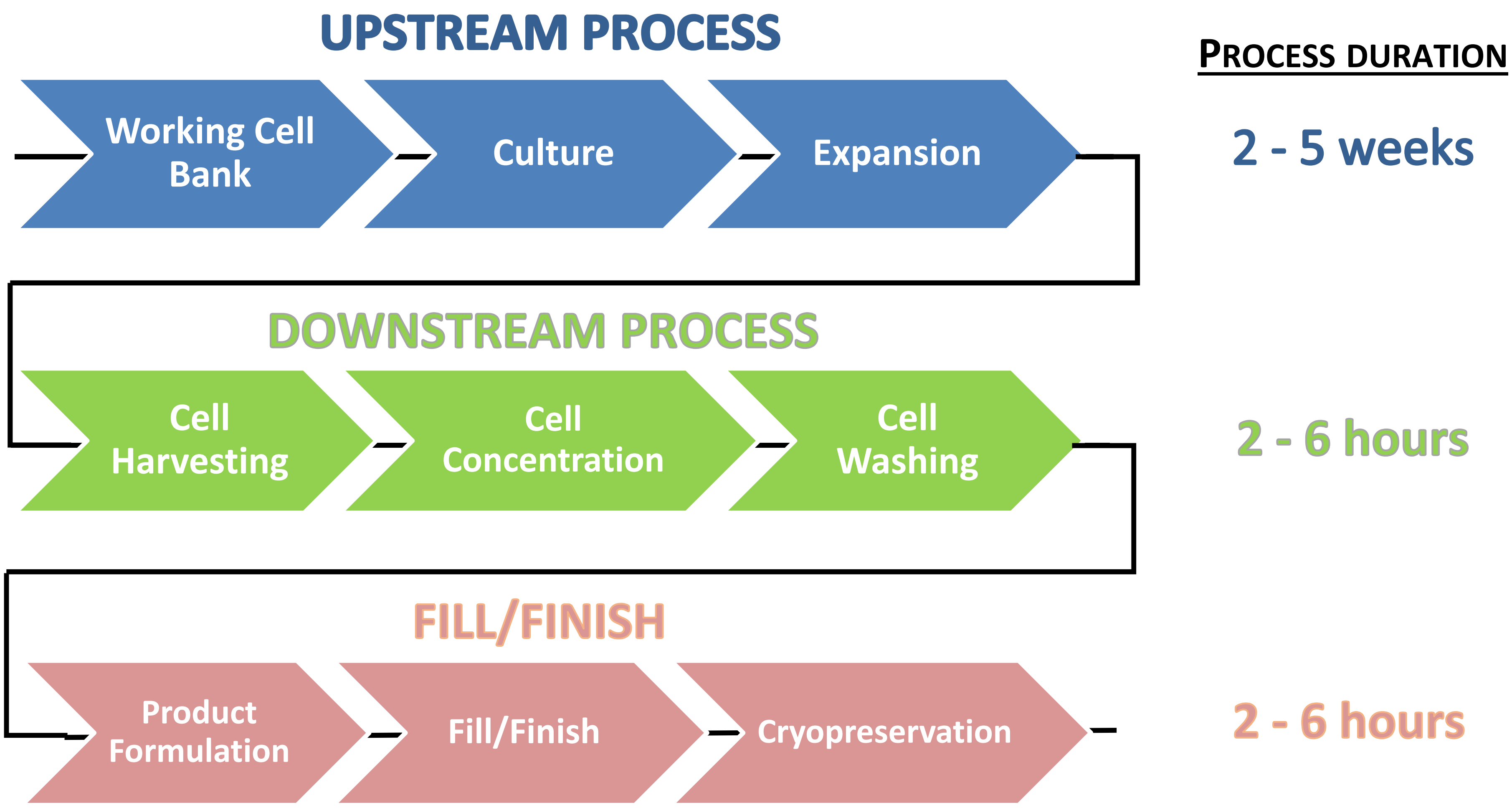


Treatment of GvHD, myocardial infarction, stroke, …

 Courtesy: G. Russotti, Celgene Cellular Therapeutics

Murphy M. et al. Exp. Mol. Medicine 2013; 45:e54

- Biomanufacturing challenges of cell-based therapies



Process time impacts “cell enviromes” and thus Cell (=PRODUCT) attributes

- BIOMANUFACTURING CHALLENGES: hMSC

Product Attributes

Identity, Potency

Purity

Process impurities (e.g. sti, BSA, microcarriers,
non-visible particles, visible foreign particles)

Safety

Sterility, Endotoxin, Mycoplasma, Viruses

Cell dose

Cell concentration, Cell viability (> 80%), Volume

FUNTIONATILTY

Analyticals

Analyticals

Analyticals

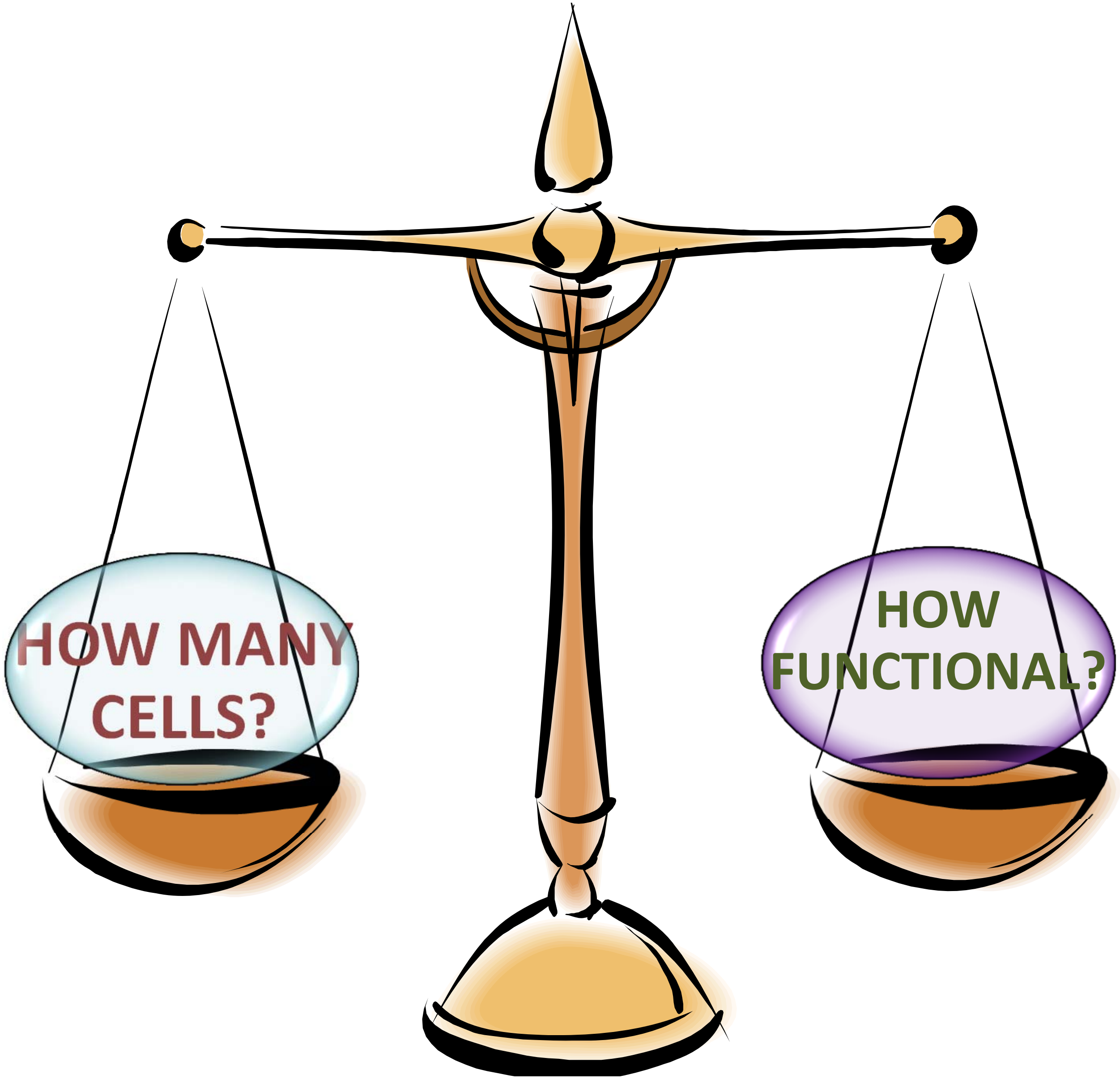
Analyticals

Analyticals

Analyticals

Tackling the bottlenecks from the beginning

Integrated *Scalable* *Robust* *GMP compliant*


HOW MANY CELLS?
HOW FUNCTIONAL?

Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells

Increase hMSC volumetric productivities using microcarrier technology and stirred tank bioreactors

Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells

Increase hMSC volumetric productivities using microcarrier technology and stirred tank bioreactors

Establish a scalable clarification process for the removal of microcarriers

- Aims

Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells

Increase hMSC volumetric productivities using microcarrier technology and stirred tank bioreactors

Establish a scalable clarification process for the removal of microcarriers

Develop filtration-based concentration and washing processes for the downstream processing of hMSC

Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells

Integration of up and downstream operations

- hMSC: Process Integration

Aiming to rationally integrate upstream and downstream operations

Upstream | **Downstream**

Cell Expansion → Harvest and Clarification → Cell Concentration → Cell Washing

Process parameters evaluated:

Cell Expansion
- Bioreactor Operation mode

Harvest and Clarification
- Membrane's material
- Pore size

Cell Concentration
- Membrane's material
- Pore size
- Initial cell concentration
- Shear rate
- Permeate flux
- Operation mode

Cell Washing
- Operation mode
- Diafiltration Volumes

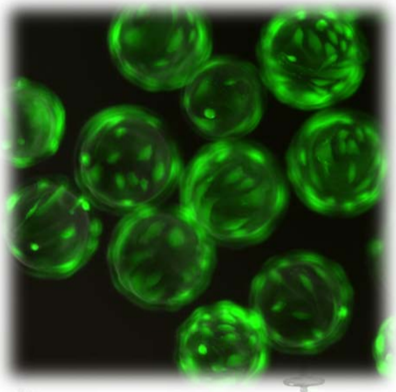
Cunha B et al 2015, Journal of Membrane Science, 478:117-129 | Cunha B et al 2015, Journal of Biotechnology, 213:97-108

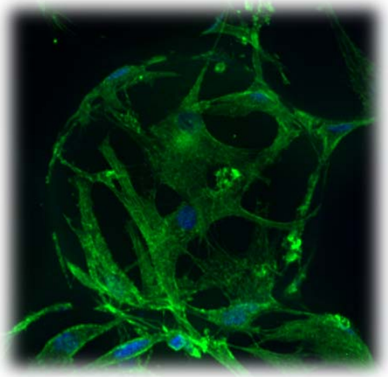
Production of hMSCs (different sources: bone marrow, adipose tissue, umbilical cord tissue)

(for Autologous and Allogeneic Therapies)

UPSTREAM BIOPROCESSING

AIM: Increase cell volumetric productivities (cell/mL) without compromising cell quality **(viability, identity and potency)**





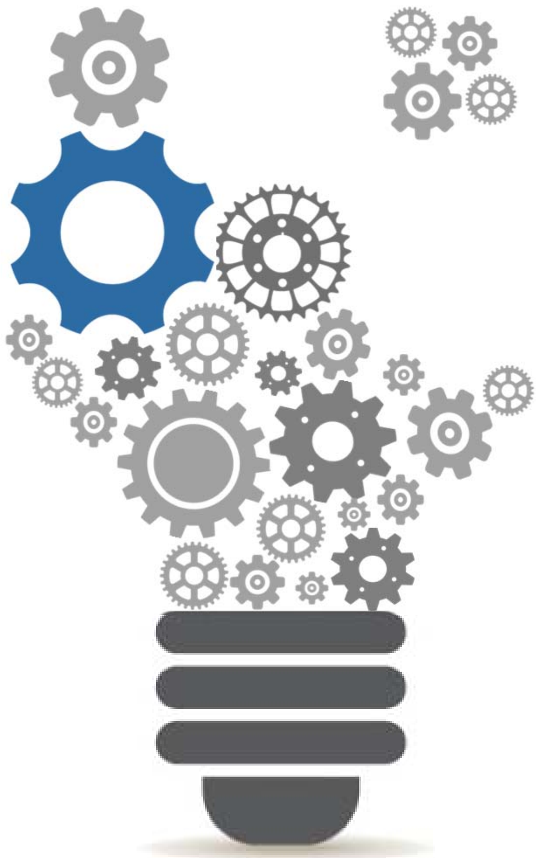
Bioprocess Development:

- Microcarrier type and concentration
- Culture operation mode (fed-batch, perfusion)
- Environmental conditions (e.g. pO_2)
-Establishment of cGMP compatible processes
- Process scale-up (from 100mL to 2L)

Sousa et al 2015, Biotechnology Progress, in press.

Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells

Cell Expansion → Harvest and Clarification → Cell Concentration → Cell Washing



Increase hMSC volumetric productivities using Microcarrier technology and Stirred Tank Bioreactors

Impact of:

Operation Mode

Semi-continuous
versus
Continuous Perfusion

on hMSC Productivity and Quality

- hMSC volumetric productivities
- hMSC metabolism
- hMSC identity and multipotency
- hMSC secretome

comparison with cells cultured in Planar Technologies (e.g. Tflasks, Cell stacks)

- USP of hMSC: Cell Expansion (Experimental Setup)

hMSC expansion in xeno-free conditions using Stirred Tank bioreactors

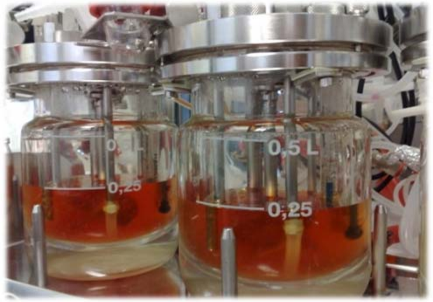
Synthetic Microcarriers: Synthemax® II (Corning)
Xeno-free Culture Medium: MesenCult-XF (Stem Cell Technologies)

Day 0 — Cell expansion → *Day 6* — Cell migration and expansion → *Day 14*

Cell inoculation + Addition of microcarriers

Addition of empty Microcarriers

Cell harvest



Operation conditions:

(V =250 mL)

- T = 37 ºC
- pO_2 = 4% O_2
- pH = 7.2

Operation Mode

SEMI-CONTINOUS

50% Medium Exchange (day 5)
50% Medium Exchange (day 7.5)
50% Medium Exchange (day 10)
50% Medium Exchange (day 12.5)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

CONTINUOUS PERFUSION

Continuous Perfusion (D = 0.2 day^{-1})

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

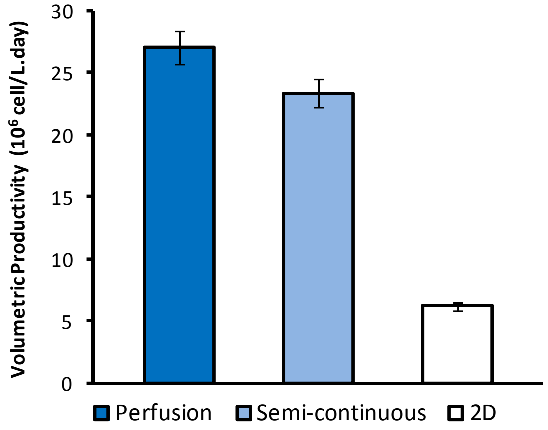
Refine TECHNOLOGY

Cell retention device:
ATF™-1 (Alternating Tangential Flow)

Cunha B et al 2015, Journal of Biotechnology, 213:97-108

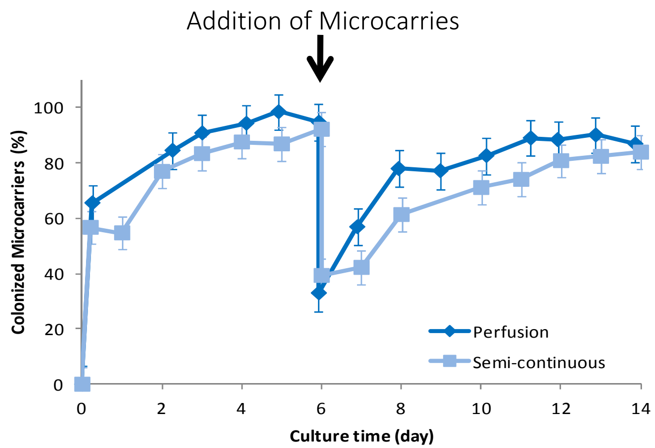
- USP of hMSC: Results

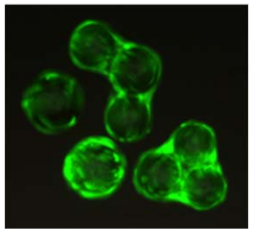
Cell Volumetric Productivities

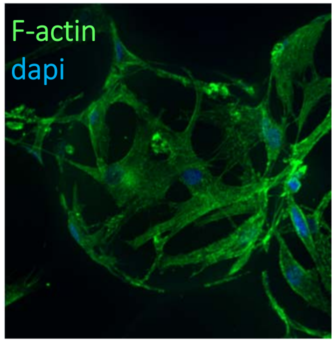


	Microcarrier-based culture in stirred tank bioreactors		Planar (2D) static culture
	Continuous perfusion	Semi-continuous	
X_{max} ($x10^5$ cell/mL)	3.4	2.8	1.0
Expansion ratio (X_{max}/X_0)	13.4	11.2	4.1

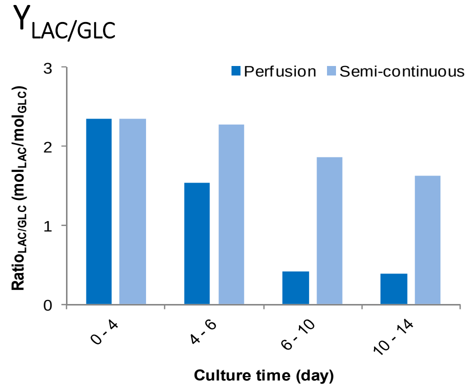
Microcarrier Colonization

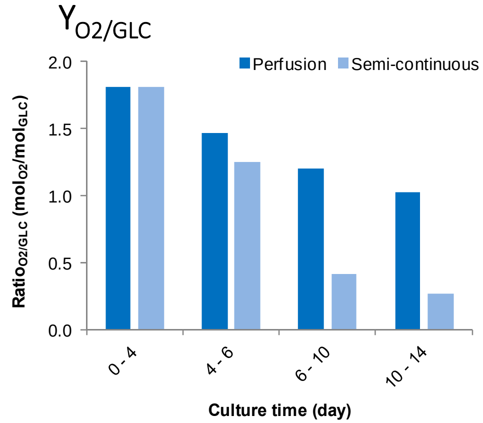






Cell Metabolism





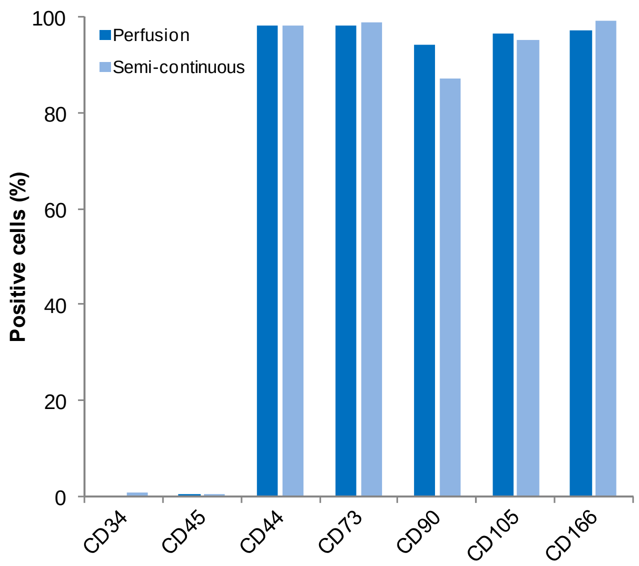
- ✓ Higher Xmax, expansion ratio and cell volumetric productivity in Bioreactors operating in continuous perfusion
  <u>5-fold improvement in cell volumetric productivities when compared to planar technologies (2D)</u>
- ✓ More efficient microcarrier colonization in Bioreactors operating in continuous perfusion
- ✓ Metabolic shift in Bioreactors operating in continuous perfusion: Anaerobic glycolysis →Oxidative phosphorilation

Cunha B et al 2015, Journal of Biotechnology, 213:97-108

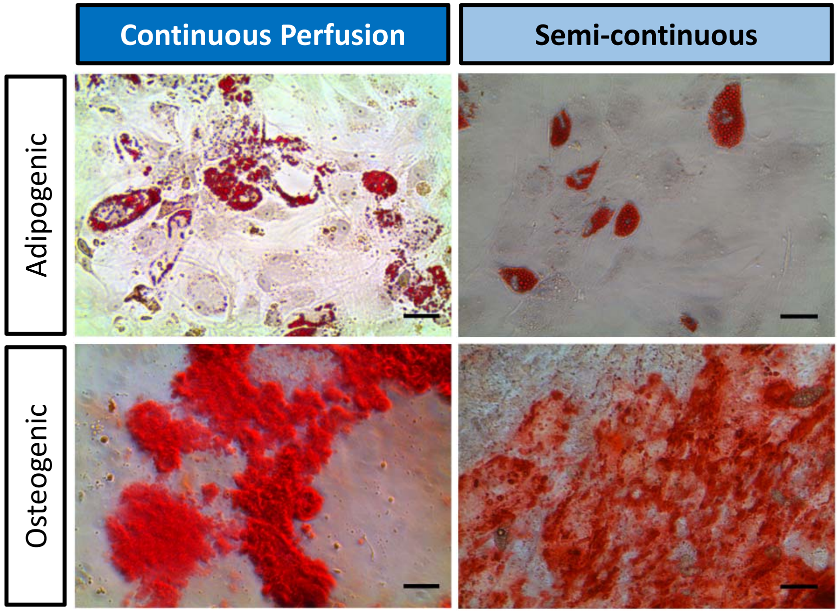
- USP of hMSC: Results

hMSC Characterization

- Identity

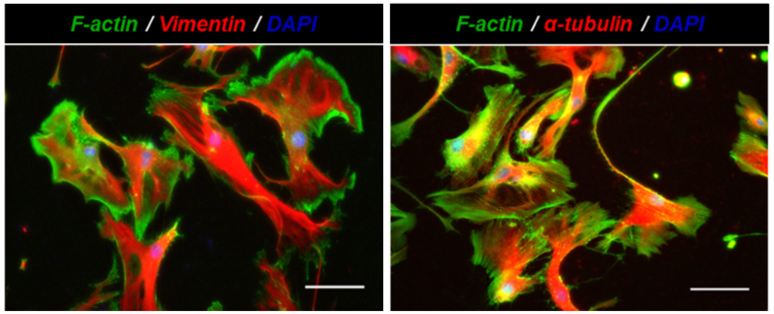


- Differentiation potential



- Morphology

hMSCs expanded in bioreactors operating in continuous perfusion



✓ **After expansion in bioreactors operating in continuous perfusion, cells maintained their identity, morphology and differentiation potential.**

✓ **No differences were observed between all culture strategies/systems evaluated**

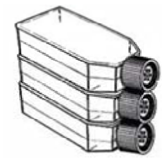
Cunha B et al 2015, Journal of Biotechnology, 213:97-108

iBET

- MASS SPECTROMETRY TOOLBOX TO CHARACTERIZE hMSC: SECRETOME

AIM: Develop a proteomics toolbox to better characterize hMSC and unveil differences in hMSC's secretome when cells are expanded in planar or bioreactor conditions

Planar



Bioreactors

Continuous Perfusion

Semi-Continuous

Cell Expansion

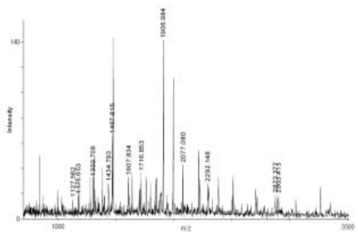
Fractionation Step

NanoLC

Mass spectrometry analysis

ESI-TripleTOF 6600 (ABSciex)

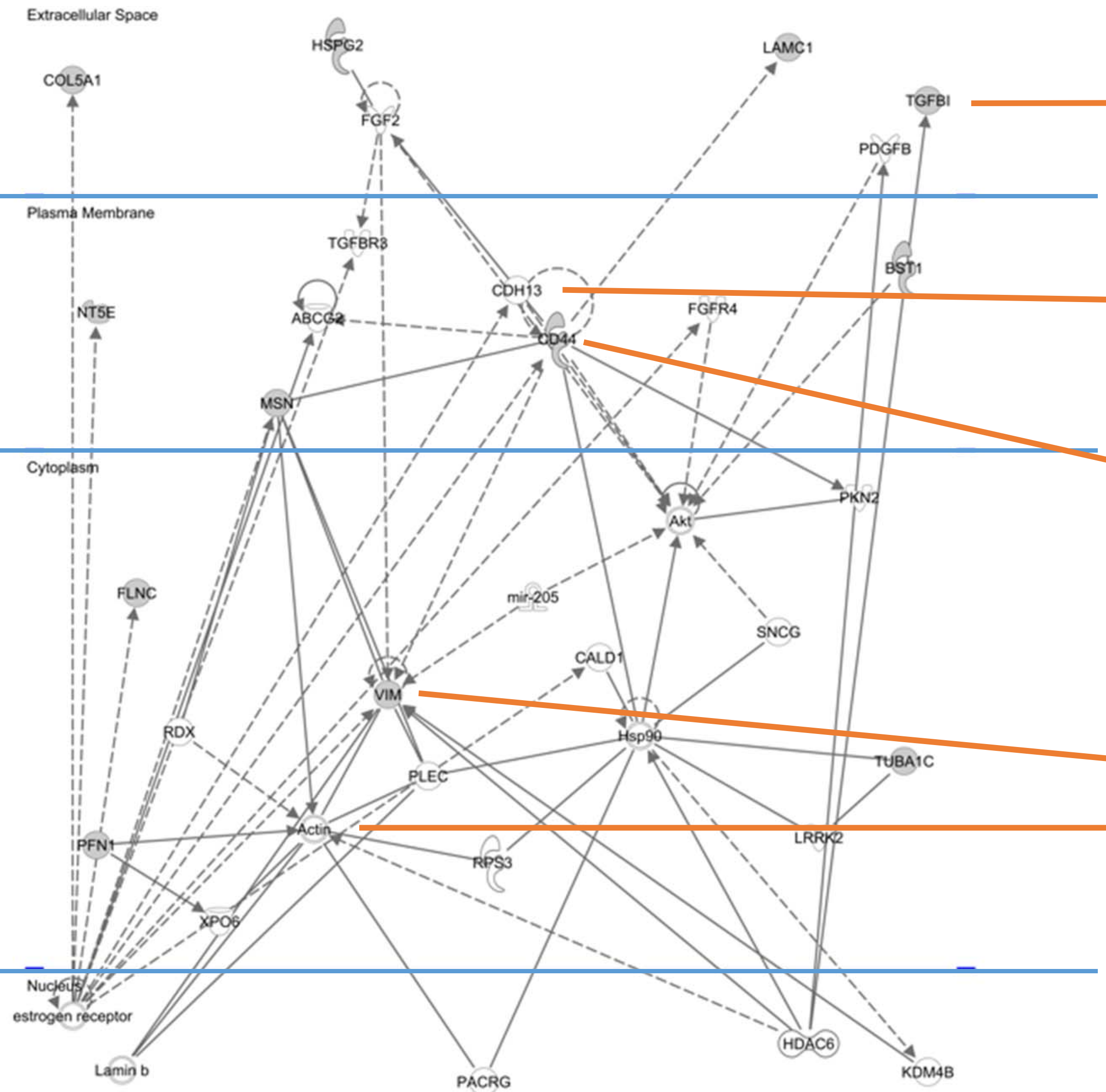
Protein Identification



- MASS SPECTROMETRY TOOLBOX TO CHARACTERIZE hMSC: SECRETOME

INGENUITY PATHWAY ANALYSIS

E.g.: Top network related with <u>Cellular Movement</u>



TGF-β1
- ✓ Controls proliferation, differentiation

Cadherin-13
- ✓ Cell adhesion

CD 44
- ✓ Involved in proliferation, adhesion, and migration
- ✓ Mediates cell-cell and cell-matrix interactions

Vimentin

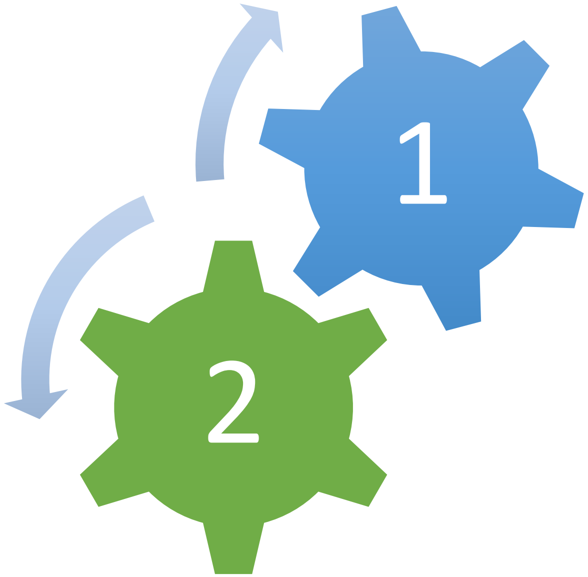
Actin
- ✓ Both involved in cytoskeleton structure and cell mobility

- USP of hMSC: Cell Expansion

1

1. Higher Xmax, expansion ratio and productivity on bioreactors (specially perfusion operation mode)

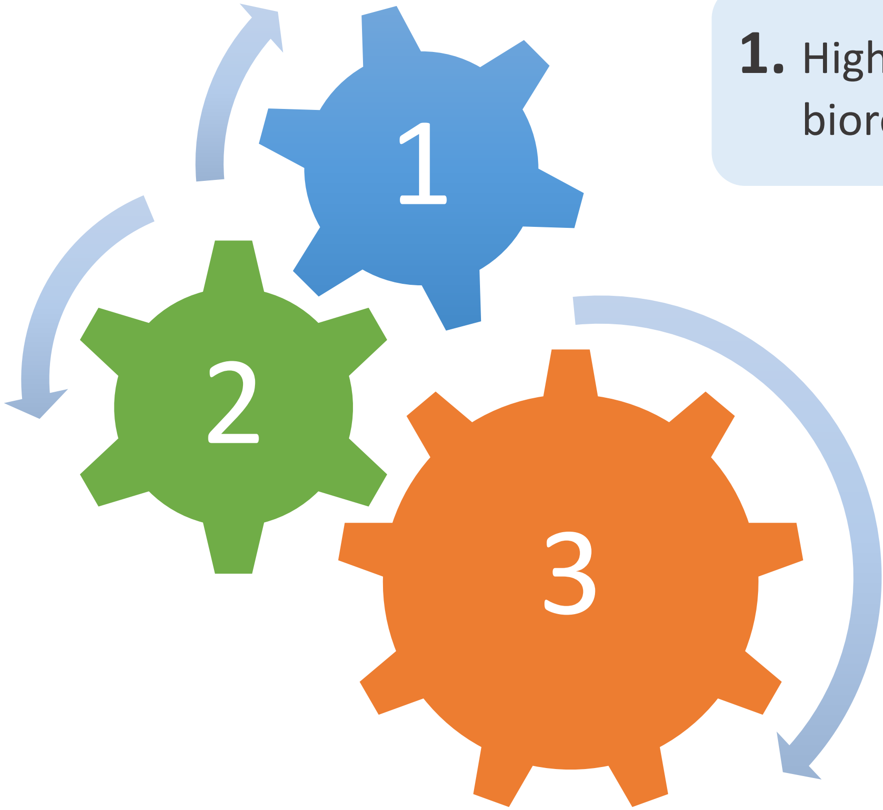
- USP of hMSC: Cell Expansion



1. Higher Xmax, expansion ratio and productivity on bioreactors (specially perfusion operation mode)

2. **Metabolic shift** in perfusion culture towards oxidative phosphorilation

- USP of hMSC: Cell Expansion

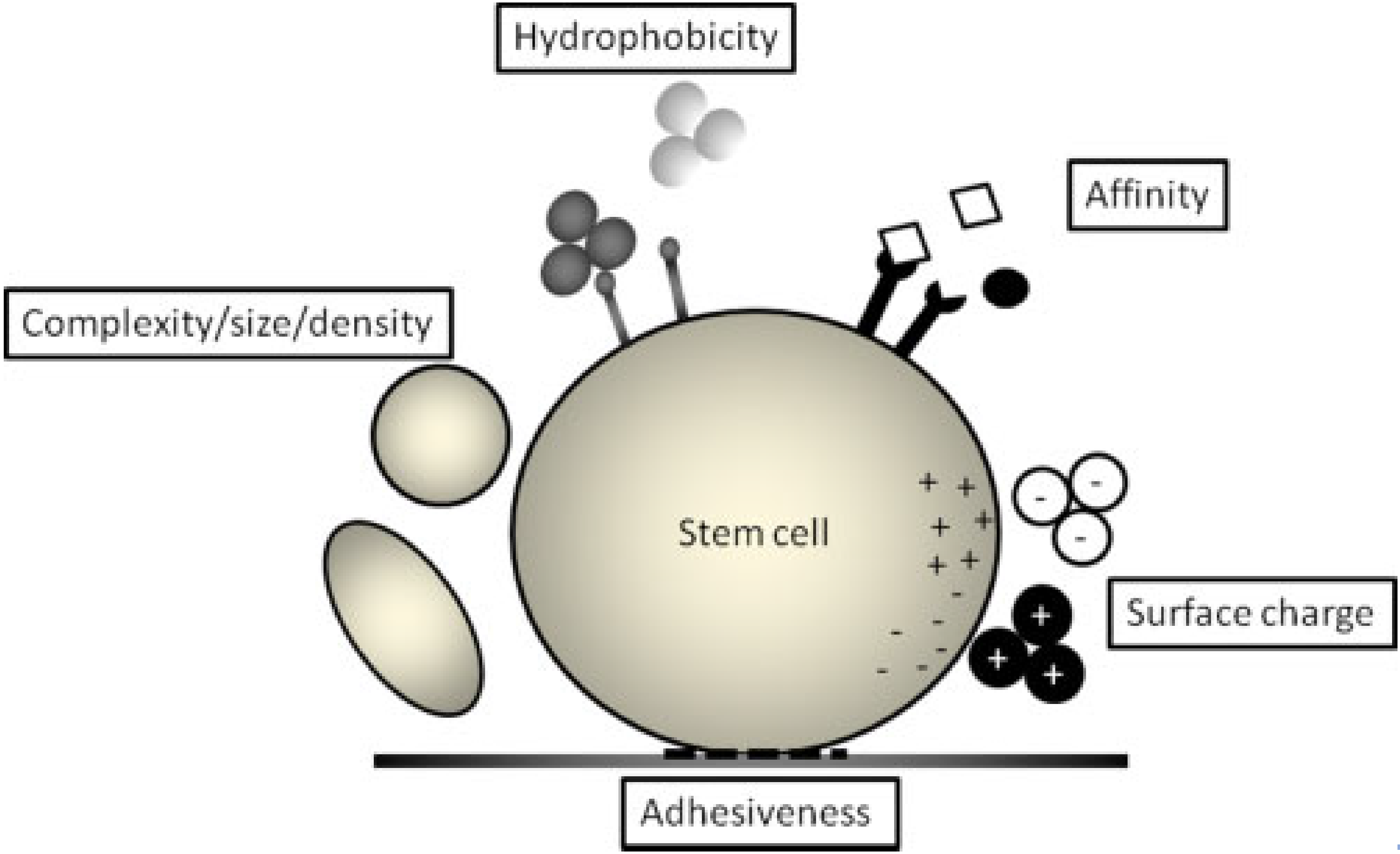


1. Higher Xmax, expansion ratio and productivity on bioreactors (specially perfusion operation mode)

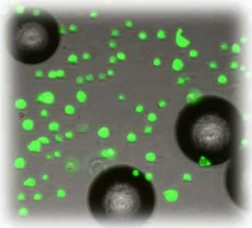
2. **Metabolic shift** in perfusion culture towards oxidative phosphorilation

3. hMSC's secretome: Bioreactors' environment leads to higher proliferation, cell migration, promote cell-to-cell signaling and representation of angiogenesis and production of growth factors – **better characterization of hMSC**

Properties of Stem Cells that can be exploited during separation process

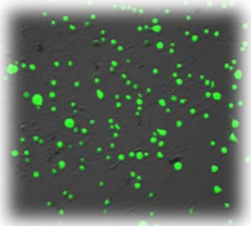


- DSP of hMSC: Clarification



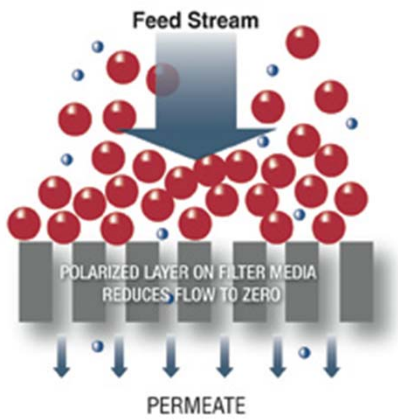
Upstream — Downstream

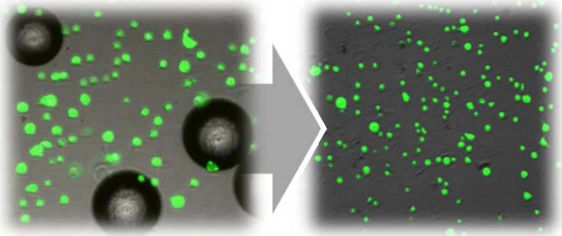
Cell Expansion → **Harvest and Clarification** → Cell Concentration → Cell Washing



AIM: Microcarrier removal

Dead-End Filtration





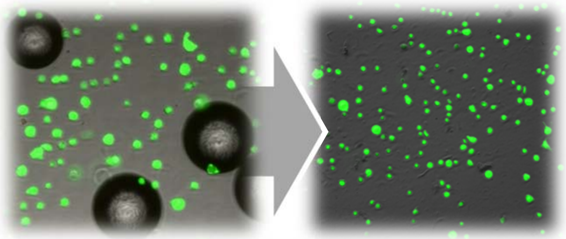
Cell Concentration = 2 x 10^5 cell/mL
Cell Size: > 15 µm
Microcarriers' size > 120 µm

- **Mesh material**
- **Pore size**

Impact on:

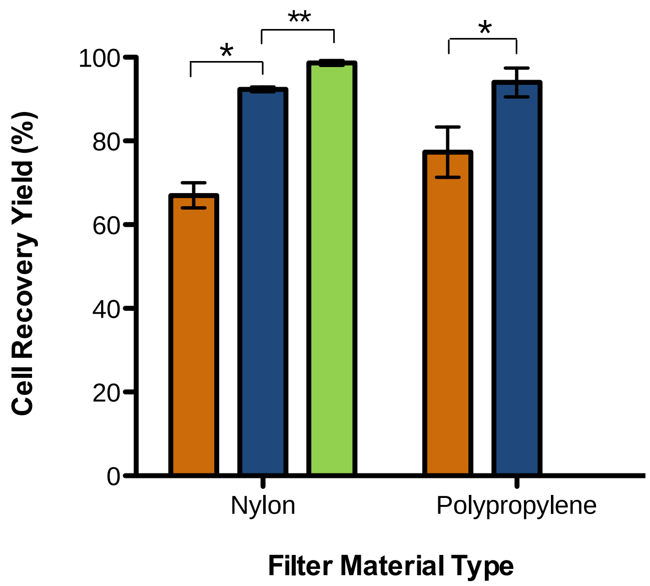
✓ **Cell viability**
✓ **Cell recovery yields**

- DSP of hMSC: Clarification

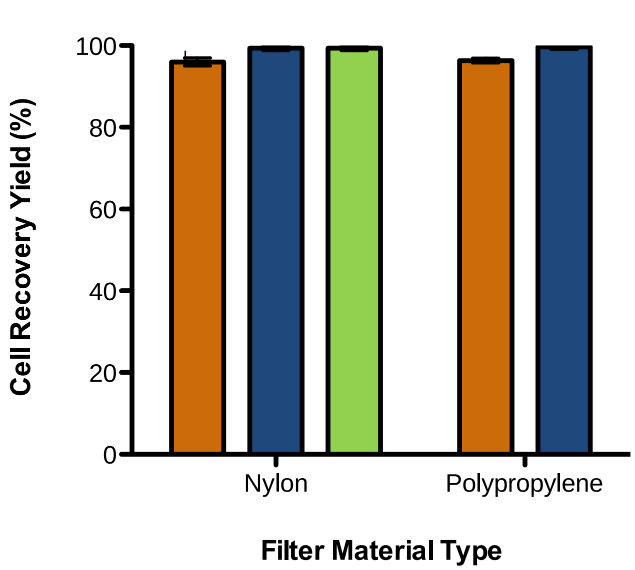


Cell Concentration = 2 x 10^5 cell/mL
Cell Size: > 15 μm
Microcarriers' size > 120 μm

Cell Recovery Yields



Cell Viability



Filter pore size:

30 μm
80 μm
100 μm

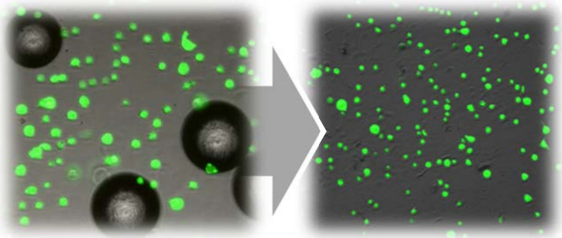
- ✓ **Higher** filter **pore sizes** yield higher cell recoveries
- ✓ **No differences** observed in cell viability (>98%) and recovery yields (>92%) **between the materials**
- ✓ **Efficient microcarrier removal** in all tested strategies (confirmed by microscopic evaluation)

Cunha B et al 2015, Journal of Membrane Science, 478:117-129 | Cunha B et al (2013) BMC Proceedings

- DSP OF hMSC: CLARIFICATION

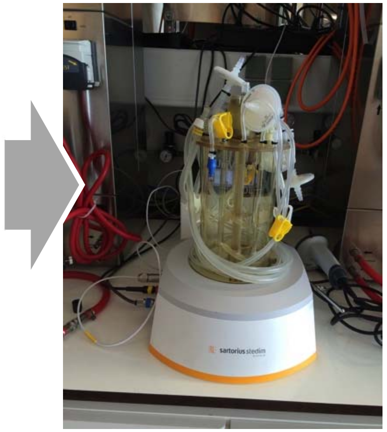
Evaluation of scalable strategies for microcarrier removal

"Scaling-up": from 0.2 to 2 L



Towards an integrated bioprocess for hMSC manufacturing

OptiCap® XL 1 Capsules (EMD Millipore)
(polypropylene, >75 µm pore size)

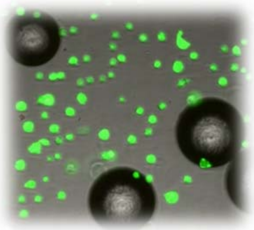


Scale	Cell Recovery (%)	Cell Viability (%)
0.2 L	> 90%	> 95%
2 L	94%	98%

✓ **Cell recovery yields and viability** were maintained after scale-up

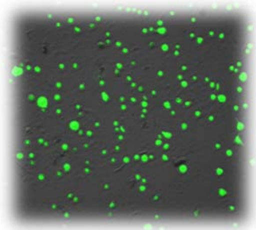
Cunha B et al 2015, Journal of Membrane Science, 478:117-129 | Cunha B et al (2013) BMC Proceedings

- DSP of hMSC: Cell Concentration



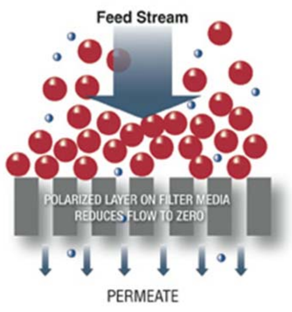
Upstream | Downstream

Cell Expansion → Harvest and Clarification → **Cell Concentration** → Cell Washing



AIM: Microcarrier removal

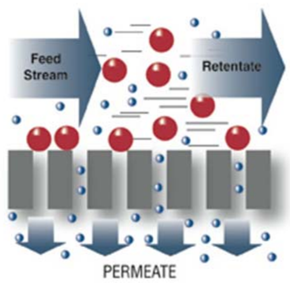
Dead-End Filtration



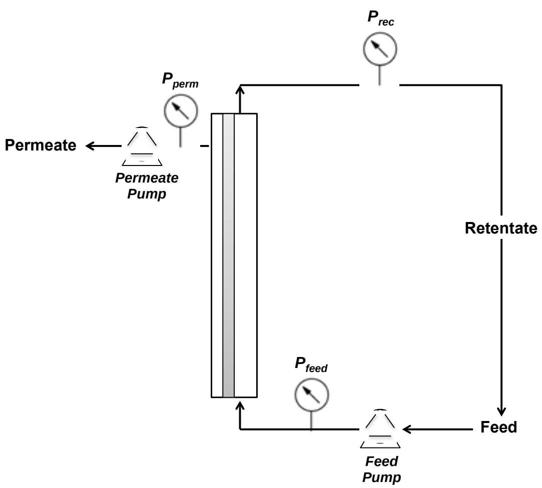
http://www.spectrumlabs.com/filtration/Edge.html

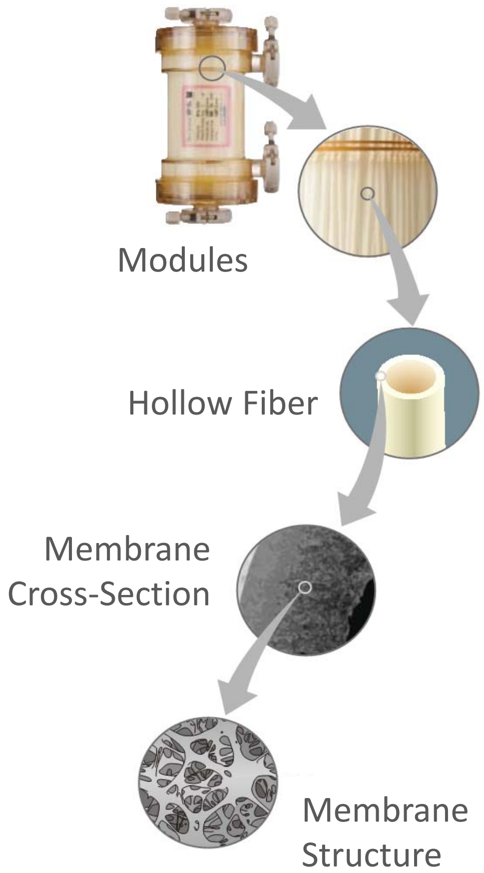
AIM: Volume Reduction

TANGENTIAL FLOW FILTRATION



http://www.spectrumlabs.com/filtration/Edge.html

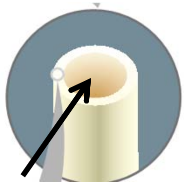




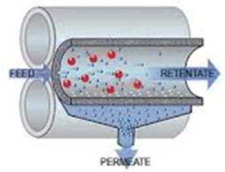
https://www.ak-bio.com

- DSP of hMSC: Cell Concentration

Impact of the shear rate on the concentration process:



ps://www.ak-bio.com



$$\gamma = \frac{4\,Q}{\pi\, r^3}$$

Inlet Flux .m^{-2}.h^{-1})	Cell Viability (%) VRF=4	Cell Rec. Yield (%), VRF=4
175	85	38
375	92	62
750	95	92

Volume Reduction Factor

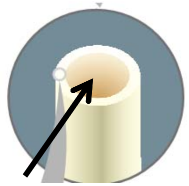
- **Higher shear rates (3000 s^{-1}):** Recovery of higher number of viable cells

Cunha B et al 2015, Journal of Membrane Science, 478:117-129

- DSP of hMSC: Cell Concentration

Impact of the shear rate on the concentration process:

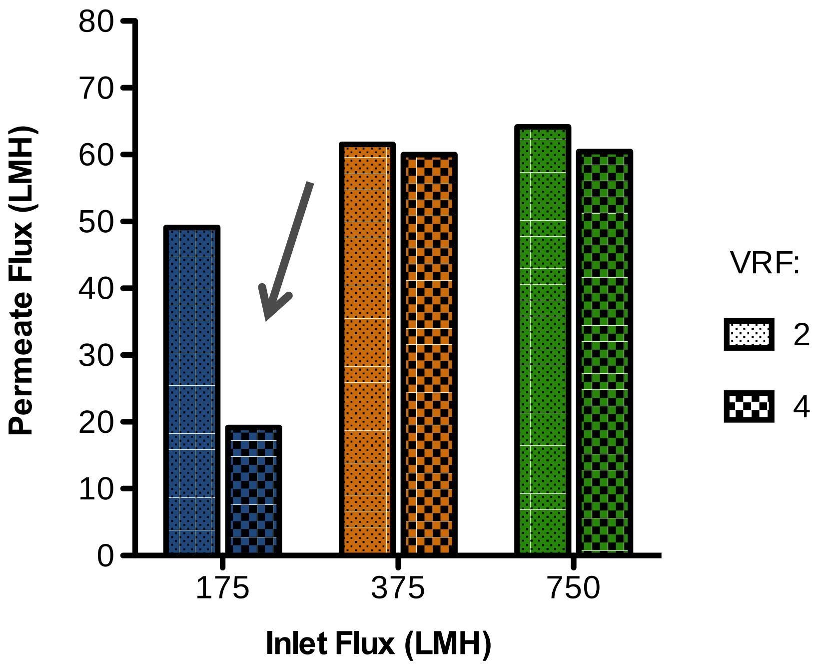
$700\ s^{-1}$ = 175 LMH $1500\ s^{-1}$ = 375 LMH $3000\ s^{-1}$ = 750 LMH



$$\gamma = \frac{4\,Q}{\pi\, r^3}$$

https://www.ak-bio.com

Permeate Flux



Cell Lysis

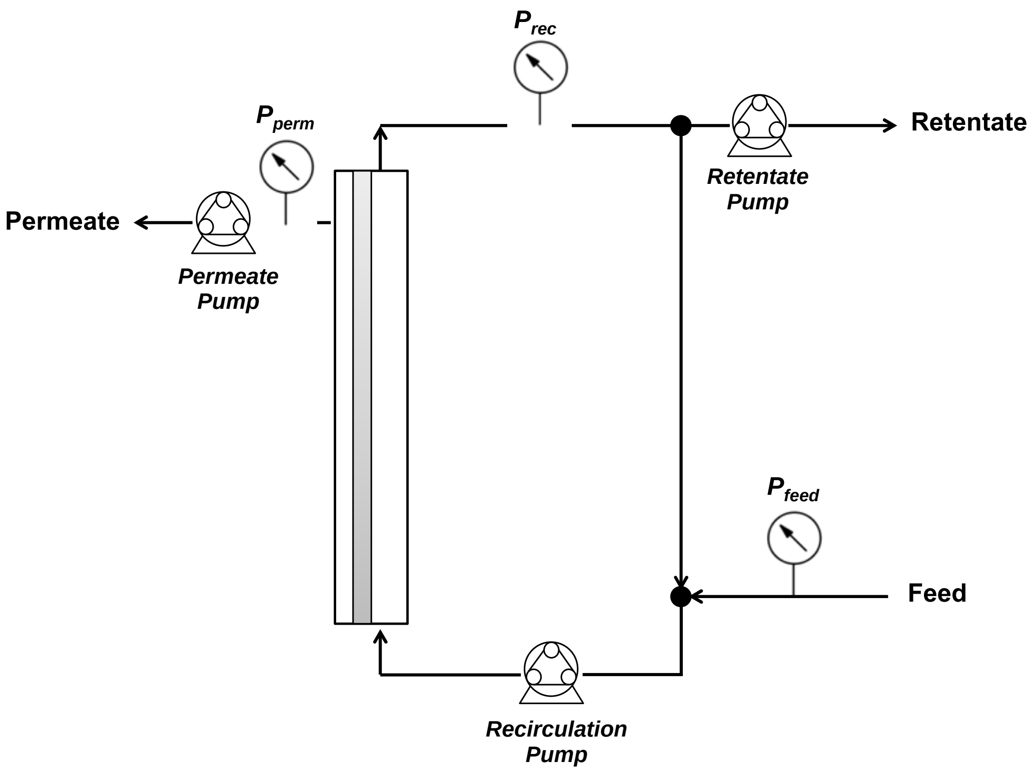
✗ **Lower shear rates ($700\ s^{-1}$):** decline on the permeate flux over time, leading to irreversible fouling and increase in cell death – higher residence time

Cunha B et al 2015, Journal of Membrane Science, 478:117-129

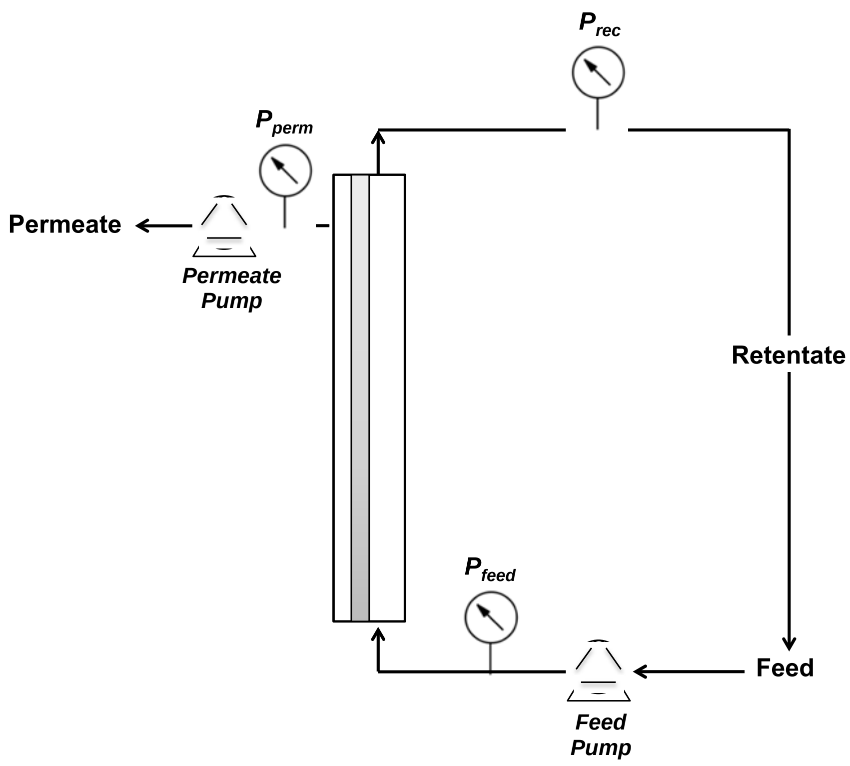
- DSP of hMSC: Cell Concentration

Impact of the operation mode (Continuous and Discontinuous TFF):

CONTINUOUS TFF

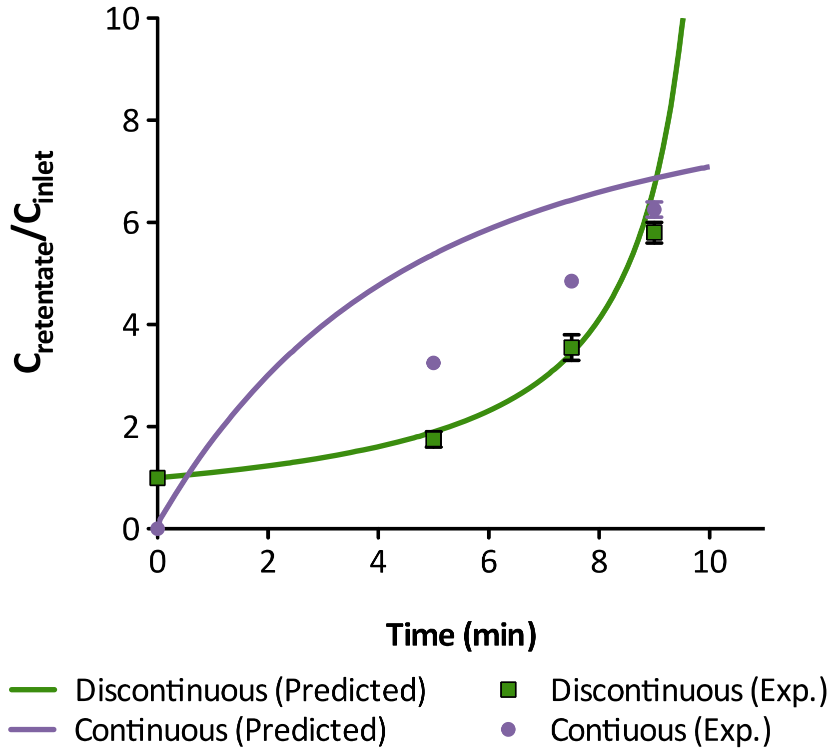


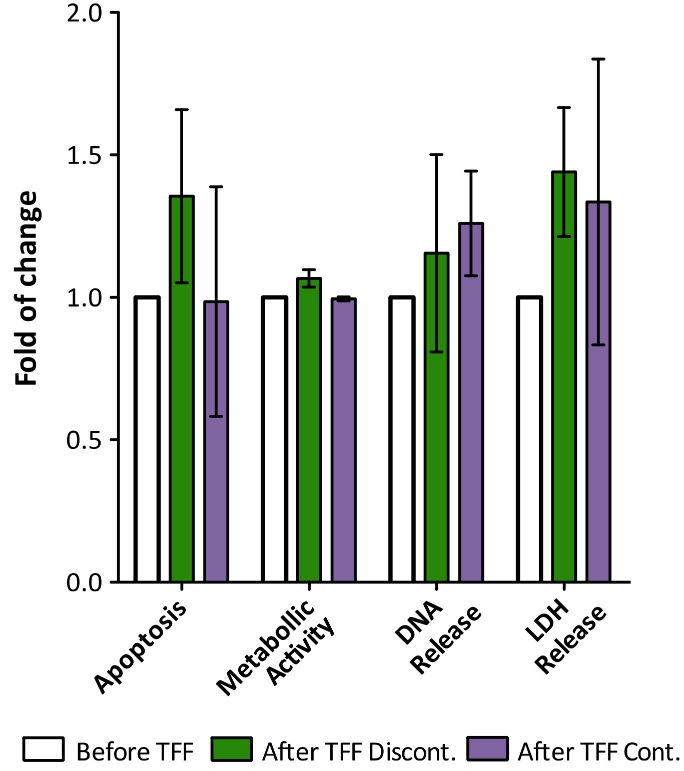
DISCONTINUOUS TFF



Cunha B et al 2015, Journal of Biotechnology, 213:97-108

Impact of the operation mode (Continuous and Discontinuous TFF):

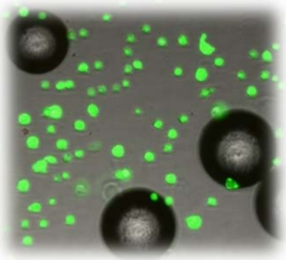


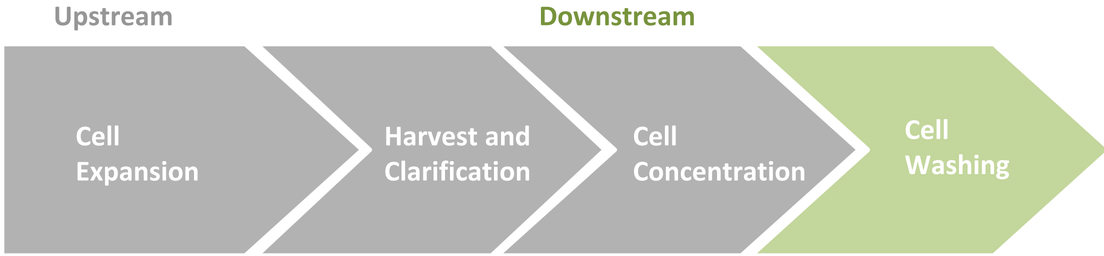


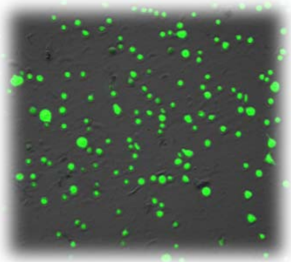
- ✓ **Both operation modes:** No impact on cell recovery yield (80%)
- ✓ **Continuous TFF:** higher concentration factors were achieved sooner however for process integration (using the same device) only discontinuous is suitable.

Cunha B et al 2015, Journal of Biotechnology, 213:97-108

- DSP of hMSC: Cell Washing

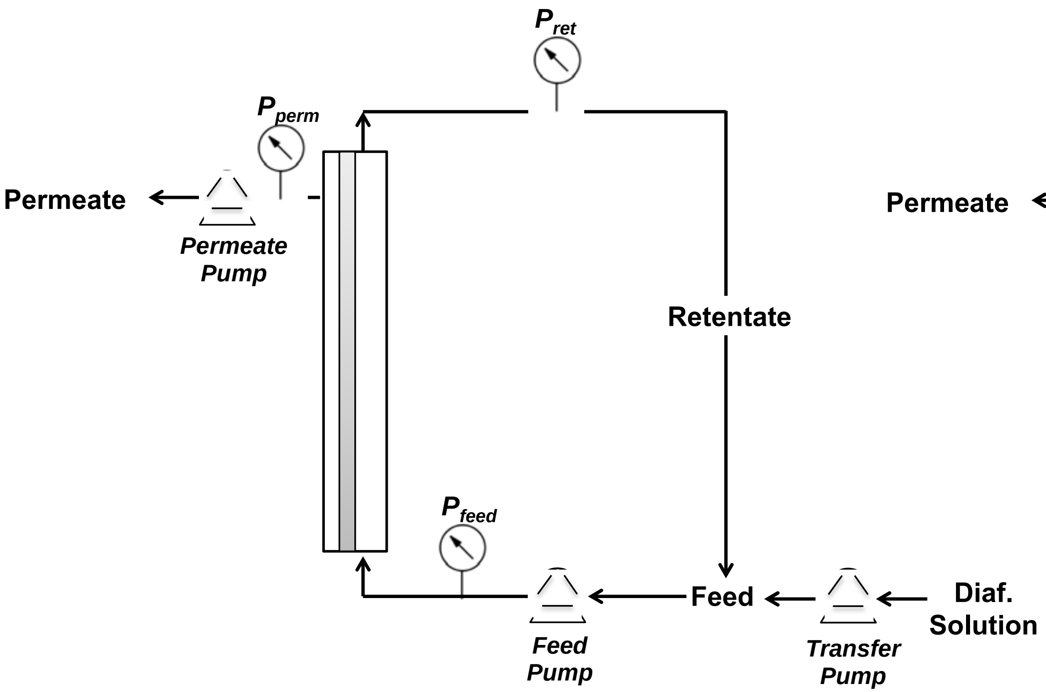




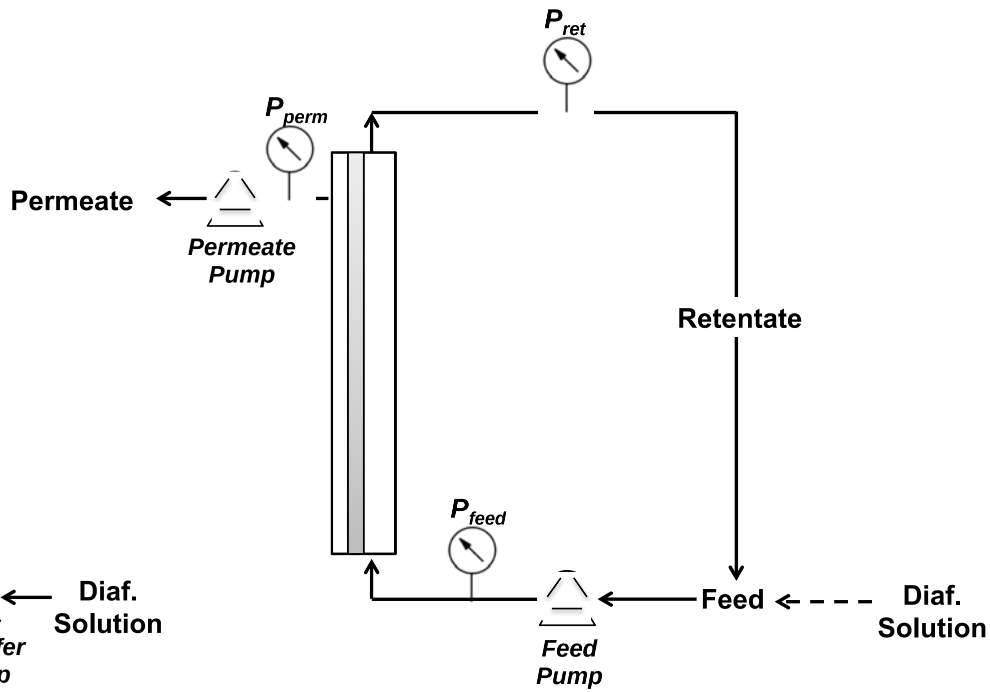


AIM: Protein Removal

CONTINUOUS DIAFILTRATION



DISCONTINUOUS DIAFILTRATION



Cunha B et al 2015, Journal of Biotechnology, 213:97-108

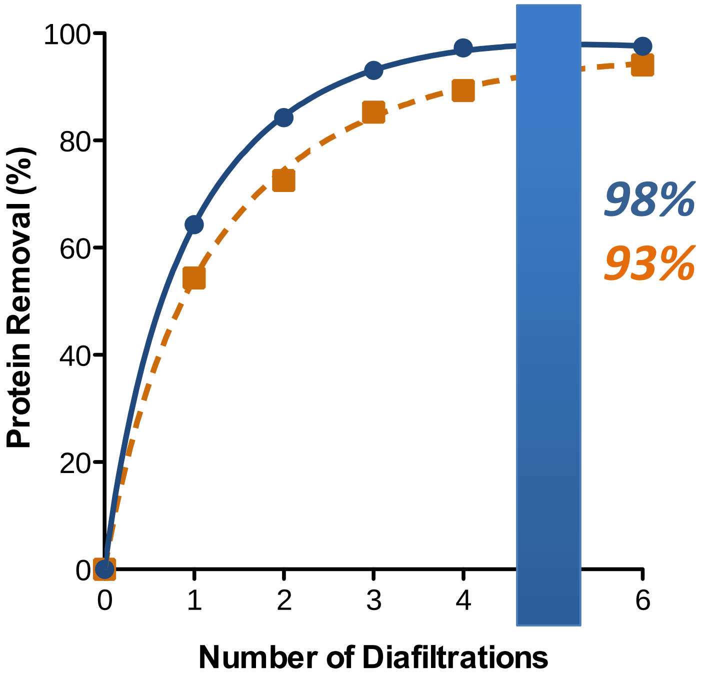
- DSP OF hMSC: CELL WASHING

Impact of different operation modes of TFF on protein removal and cell recovery yield (high protein clearance, high cell recoveries, high viability, QA)

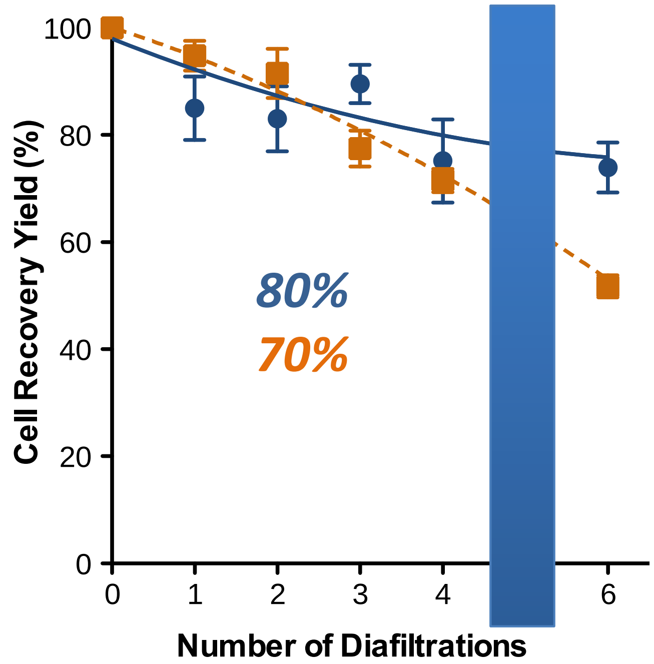
CONTINUOUS DF
DISCONTINUOUS DF

Concentrated cell suspension
DMEM + 10% FBS
DF with DPBS

Protein Clearance



Cell Concentration



✓ **Continuous Diafiltration:** Higher protein clearance (98%) and cell recovery yield (80%)

Cunha B et al 2015, Journal of Biotechnology, 213:97-108

- hMSC: Process Integration

Aiming to rationally integrate upstream and downstream operations

Upstream | **Downstream**

Cell Expansion → Harvest and Clarification → Cell Concentration → Cell Washing

Cell Expansion
- **Operation mode**
  Continuous Perfusion

Harvest and Clarification
- **Membrane's material**
  Polypropylene
- **Pore size**
  > 75 µm

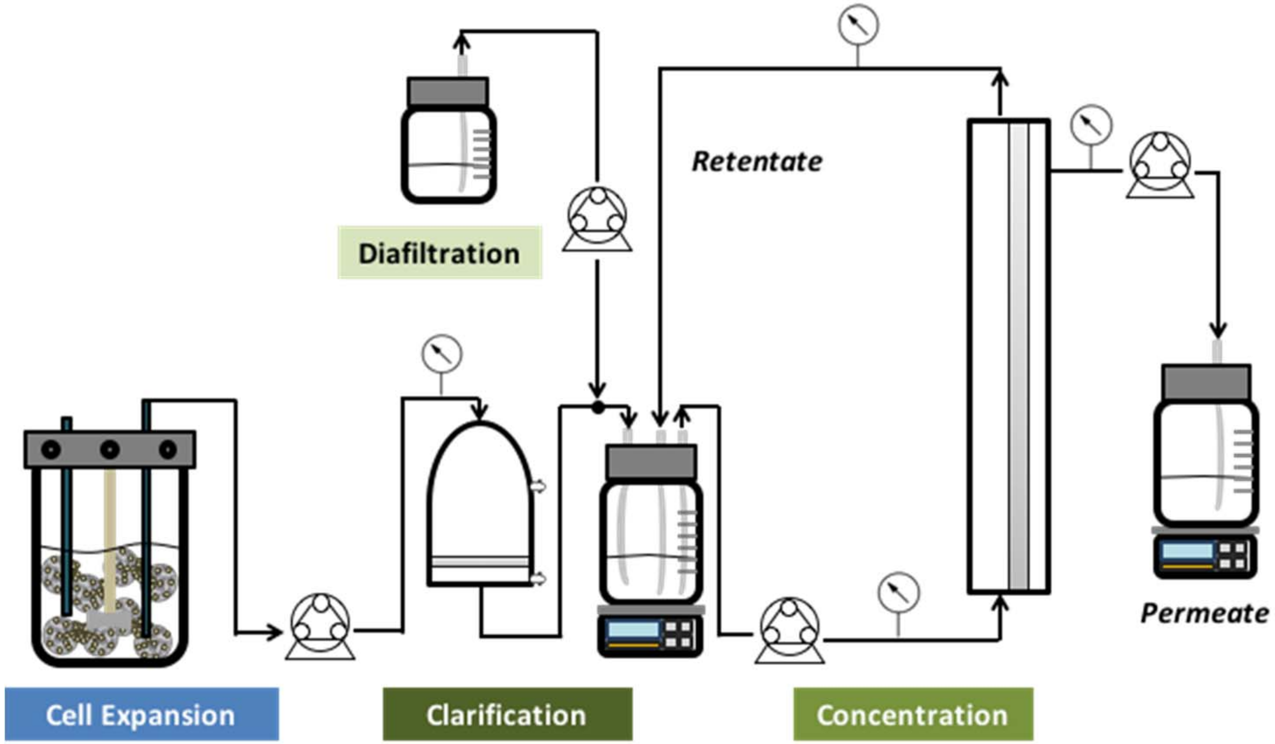
Cell Concentration
- **Membrane's material**
  Polysulfone
- **Pore size**
  > 0.45 µm
- **Initial cell concentration**
  > 2 x 10^5 cell/mL
- **Shear rate**
  3000 s^{-1}
- **Permeate flux**
  250 LMH
- **Operation mode**
  Discontinuous TFF

Cell Washing
- **Operation mode**
  Continuous DF
- **Diafiltration Volumes**
  5 DVs

Cunha B et al 2015, Journal of Membrane Science, 478:117-129 | Cunha B et al 2015, Journal of Biotechnology, 213:97-108

- hMSC: Process Integration

Aiming to rationally integrate upstream and downstream operations



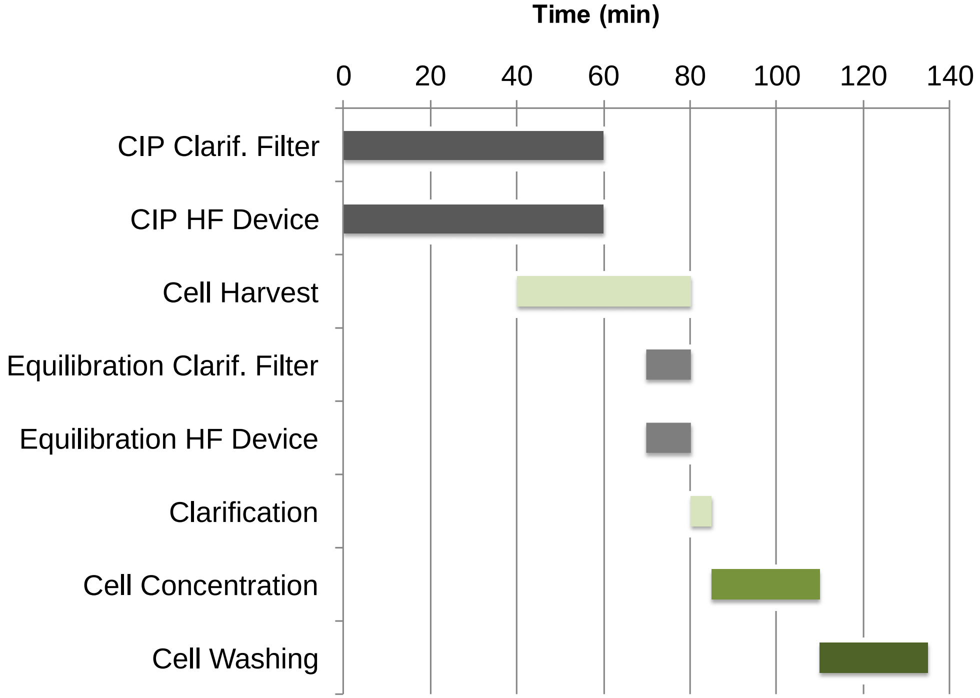
Integration:

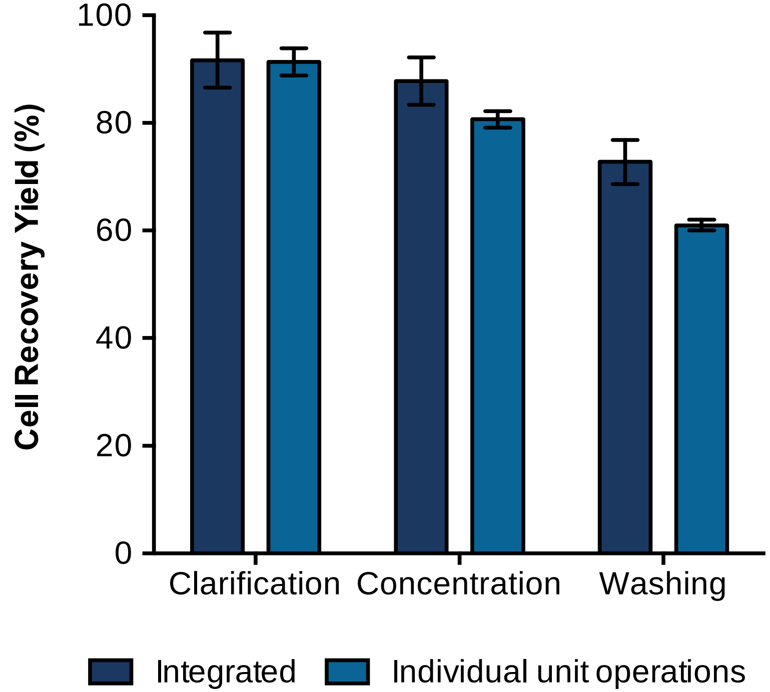
Closed system, resulting in the elimination of hold steps and decreasing the equip. footprint

Cunha B et al 2015, Journal of Biotechnology, 213:97-108

- hMSC: Process Integration

VRF = 10X; 5 DVs

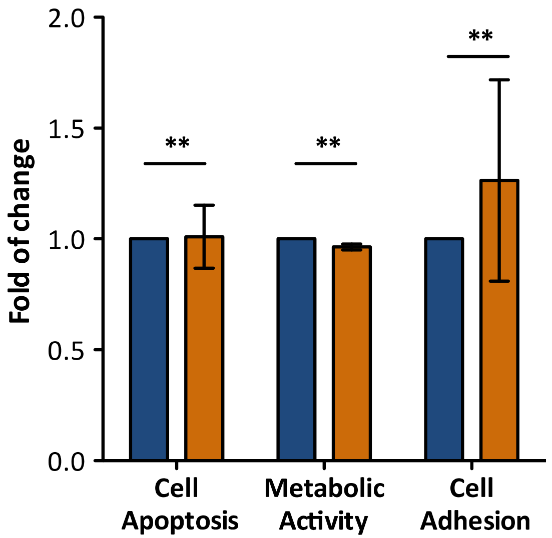


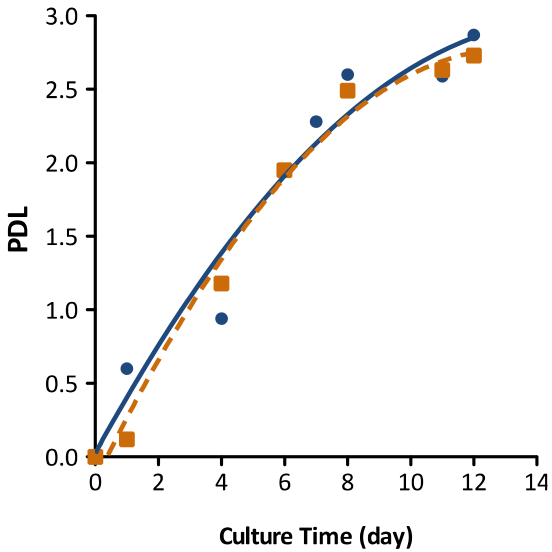


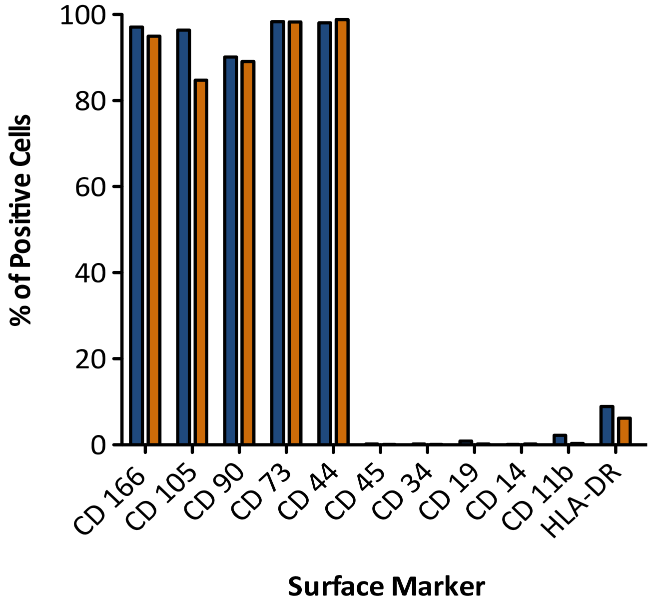
- ✓ **Faster** processing after cell expansion (1,5 h)
- ✓ **Cell Recovery Yield:** More than 70% of hMSC, with viability > 95% (10% more than having separate operations)

- hMSC: Process Integration – Quality Assays

After USP
After DSP





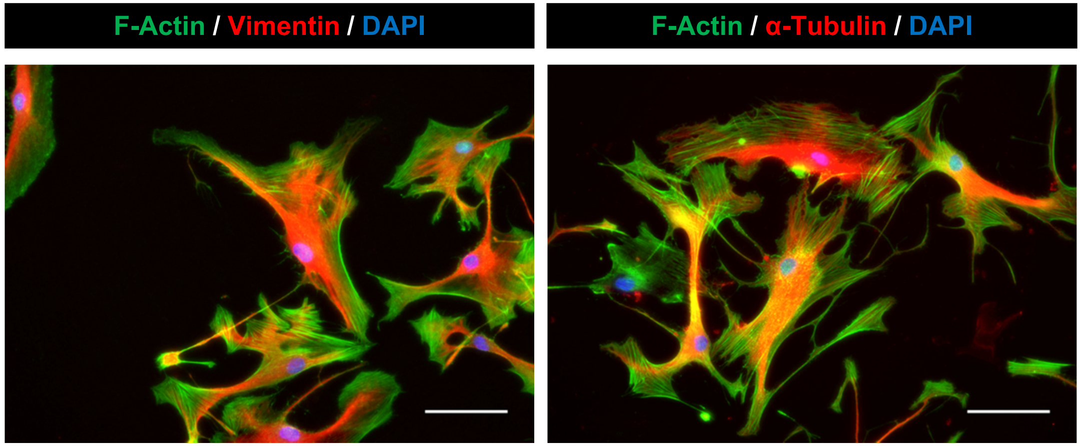


- ✓ hMSC maintained their immunophenotype and metabolic activity after processing;
- ✓ Ability to adhere to plastic surfaces and proliferative capacity (PDL = 3) after re-plating

Cunha B et al 2015, Journal of Biotechnology, 213:97-108

- hMSC: Process Integration – Quality Assays

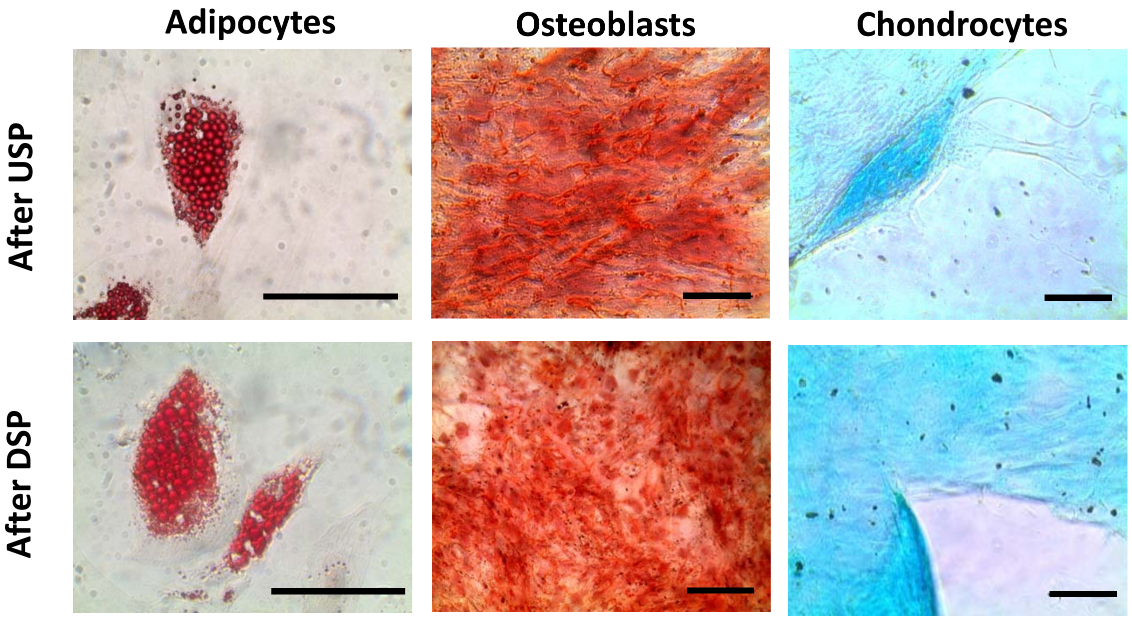
Morphology



Morphology and adhesion:

hMSC successfully re-acquire their typical spindle-like morphology with organized actin fibers

Differentiation Potential

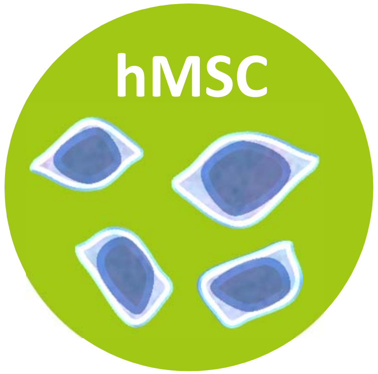


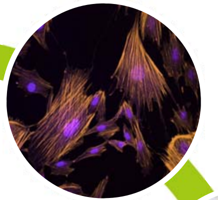
Potency:

hMSC maintained their multilineage differentiation potential

Cunha B et al 2015, Journal of Biotechnology, 213:97-108

- DSP OF hMSC: ACHIEVEMENTS





Product Attributes

Identity, Potency

Identity, potency, morphology, proliferation, metabolic activity

Purity

Process impurities (e.g. BSA, microcarriers, non-visible particles, visible foreign particles)

98% removal of protein impurities, no microcarriers

Safety

Sterility, Endotoxin, Mycoplasma, Viruses

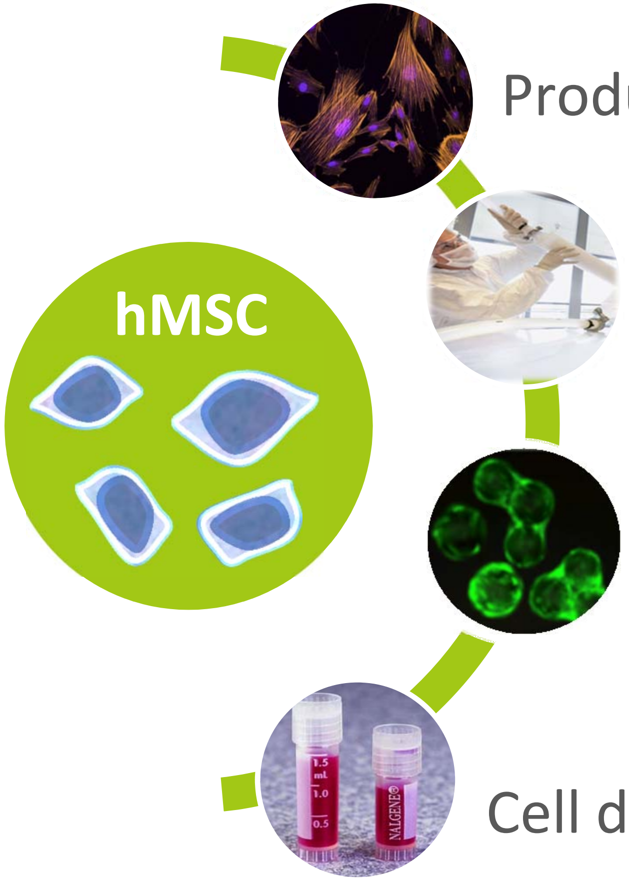
Sterility throughout processing; endotoxin-free; single-use material

Cell dose

Cell concentration, Cell viability (> 80%), Volume

VRF up to 10X; 70% cell recovery; 95% cell viability

- DSP of hMSC: Achievements



Product Attributes

Purity

Safety

Cell dose

Tackling the bottlenecks from the beginning

Robust

Scalable

Integrated

Clinical-grade

DSP:

- ✓ Applicability for both autologous and allogeneic therapies (scale-out and scale-up)
- ✓ Valid for other stem cell types relevant for the cell therapy industry (e.g. hiPSC)

iBET

- ACKNOWLEDGEMENTS

Animal Cell Technology Unit

www.ibet.pt/research/animal-cell-technology

Animal Cell Technology

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Thank you

"The cell therapy industry has an opportunity to learn from protein processing and anticipate Upstream & DSP bottlenecks by proactively developing technologies to address future scales."

Adapted fromPattasseril et al (2013)